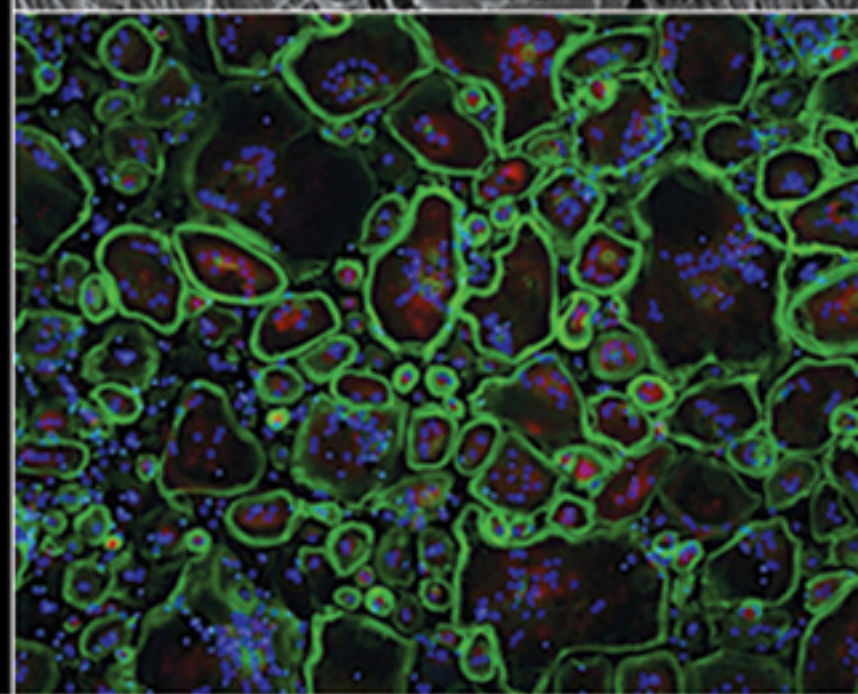
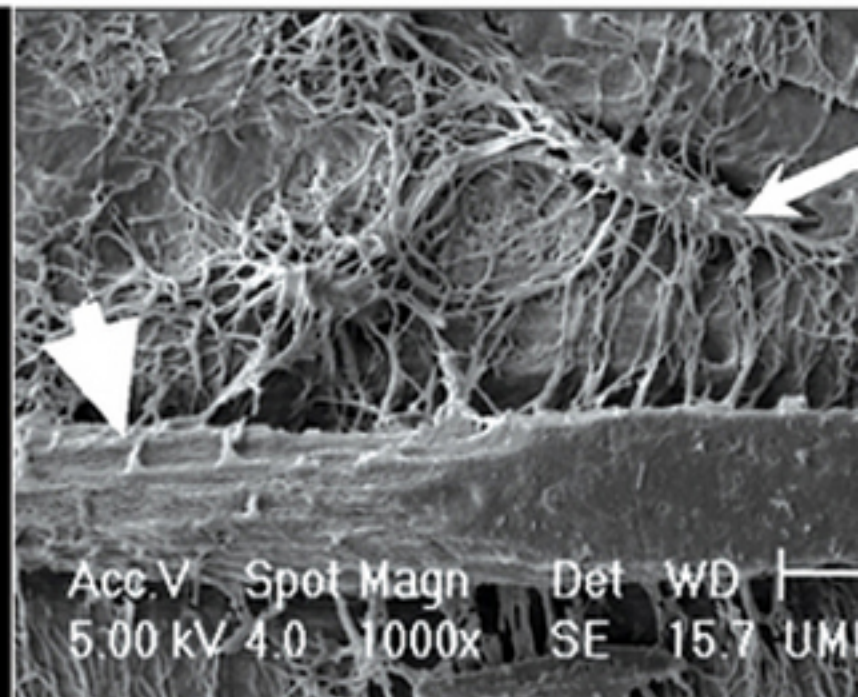
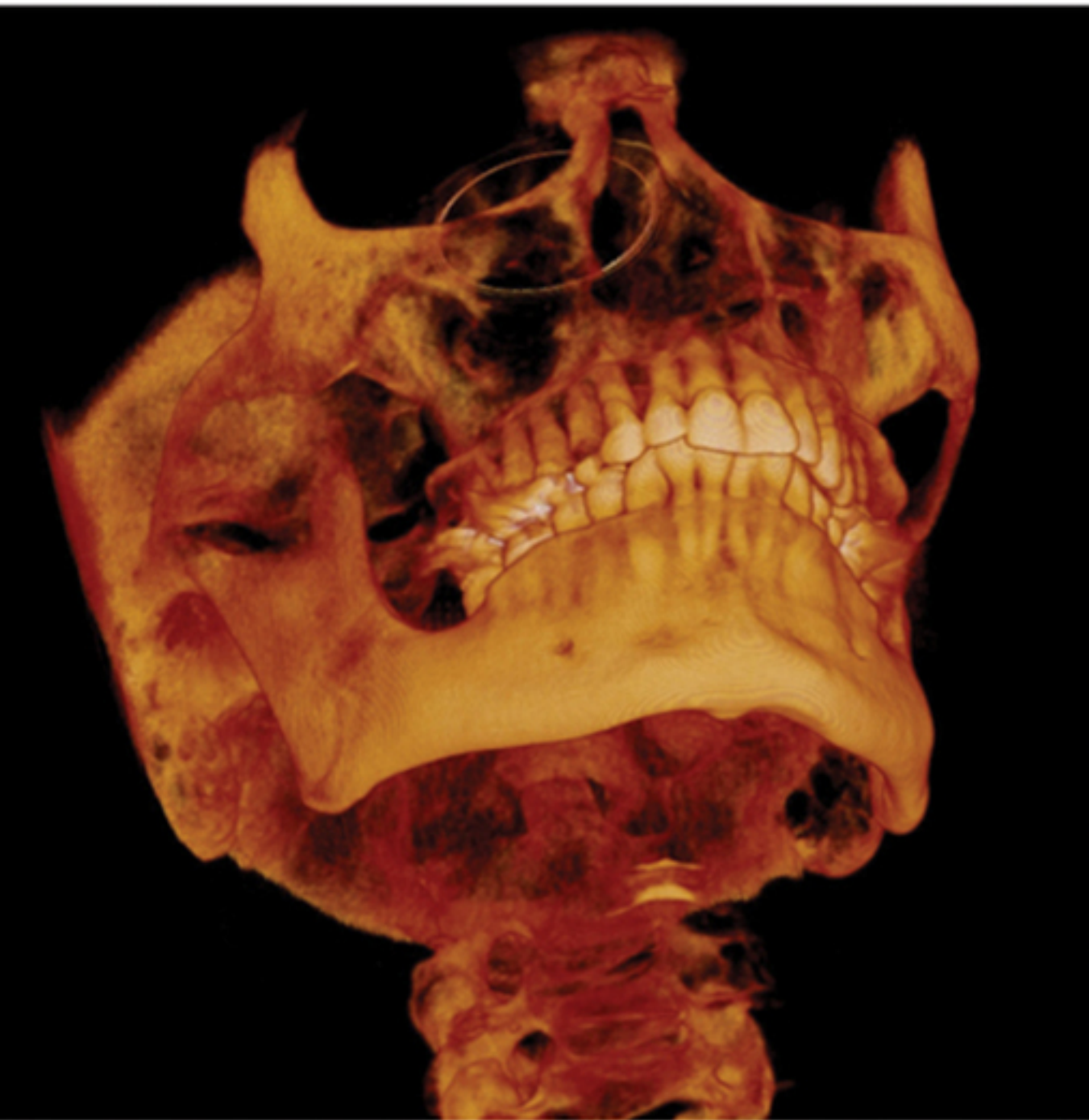


Mineralized Tissues in Oral and Craniofacial Science

Biological Principles and Clinical Correlates



Edited by
Laurie K. McCauley & Martha J. Somerman



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Figures from the book are available for download at www.wiley.com/go/mccauley

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Preface

The idea for this book was conceptualized in 2009, at an annual American Academy of Periodontology meeting in Boston, which we were invited to present a continuing education symposium on mineralized tissues. Specifically, we were asked to gear our presentations to relevance for practitioners. The session was well attended and the audience was clearly interested in grasping the underlying biology of mineralized tissues of the dental-oral-craniofacial apparatus, yet with application to clinical scenarios. After the symposium and a long discussion while walking the streets of Boston, along with numerous phone calls and e-mails, the goals and objectives of this work took shape, and the colleagues who agreed to join and provide their valuable knowledge and experience made the project feasible.

The broad objective of this book is to provide a comprehensive update on knowledge in the field of mineralized tissues, focusing on the dental-oral-craniofacial region and including clinical correlates that reinforce the significance of the scientific knowledge to clinical diagnoses and therapies. Basic science chapters are followed with at least one correlate chapter of clinical relevance (i.e., case studies). To ensure a link between these, the basic and clinical correlates follow a general schematic that was largely utilized by all authors. All figures are digitized and downloadable for presentation purposes. Clinical case studies are described in a manner that lends easily to their use in teaching venues.

This original approach, linking the basic principles of hard-tissue cell and molecular biology to clinical correlates, aims to attract a diverse audience, both students and faculty, including those at early stages of their research career, as well as more senior faculty interested

in a comprehensive text for reference. Moreover, by providing clinical correlates, this text will appeal to nondental faculty and students by providing additional insights to the translational aspects of their research and also as an important reference source for students in a wide variety of healthcare programs. Finally, we anticipate interest in the textbook on the part of all health care providers who seek to understand the underlying biology of mineralized tissues they treat daily in their practice. With the exponential growth of scientific information, there is a greater need than ever before to make sure that the research communities are updated on the most current findings in all areas of science. At present, there is no comprehensive review of the topics presented here (i.e., one focusing specifically on hard tissues of the oral cavity). Equally important is the link of basic principles to clinical situations. More than ever before, as we are confronted with discoveries resulting in increasingly complex issues in science, there is a need for collaborative efforts across all disciplines in order to reach our ultimate goal of improving the quality of life for all in our community.

We enjoyed the development and orchestration of this volume tremendously. Our author colleagues were wonderfully responsive and ardently involved in their chapter contributions. The joining together of colleagues from all over the world and in all facets of this subject was highly rewarding, and we truly hope the readers will appreciate the depth and breadth this work provides.

*Laurie K. McCauley
Martha J. Somerman*

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We would like to express our appreciation to the dedicated author contributors of this book for their enthusiasm toward the approach taken to link the basic biology with clinical practice and for their shared expertise and meticulous and timely efforts to bring this to fruition. Special thanks go to Norman Schiff for coordinating the authors, making sure manuscripts were received in a timely fashion, and for his patience along the way; to Jessy Grizzle for being a publishing role model and ever patient spouse; to Dr. Erika Benarides for the CT cover

image; and to Kathy Ribbens for her assistance in editing and preparing the complete initial draft. Finally, we would like to thank the publishers for engaging in our vision to develop a book that will serve the community of scientists, scholars, teachers, clinicians, and students who seek expert information regarding craniofacial skeletal health and disease.

L.K.M.

M.J.S.

Foreword

When solid research blends with clinical application: a book for a diverse audience emerges

The craniofacial skeleton provides critical protection for the neural system and houses our precious sensory organs of sight, sound, smell, and taste. Teeth comprised of three unique mineralized tissues are supported by bone, a fourth distinct tissue. Each of these tissues has a very unique molecular and biologic profile. Bones of the oral cavity are impacted by a wide variety of infectious agents, are subject to unique biomechanical forces, and are highly responsive to environmental stresses. Virtually all of these topics are covered in this new book, edited by two preeminent clinician scientists. The subject matter is presented with a focus and depth consistent with a rigorous scientific periodical. Importantly, information is not presented in isolation, but instead flows seamlessly with excellent integration and connection to systemic interactions and clinical implications.

This new body of work orchestrated by Drs. McCauley and Somerman brings together 85 outstanding contributors from 13 countries in 39 chapters that cover all the relevant aspects of mineralized tissues pertinent to oral and craniofacial biology in health and disease. A

review of the developmental, molecular, and cellular aspects of bones and teeth sets the framework for this volume. The expert basic science reviews are enhanced further by including relevant clinical examples that speak to the strong translational focus of this book. This book will provide readers with basic tenets, recent advances, and meaningful links that impact patient care. A wide audience will benefit, including those already established in the field, new investigators, students, dental clinicians, and health care professionals in complementary areas such as endocrinology, rheumatology, orthopedics, and pediatrics, among others. We fully anticipate that this book will represent a landmark contribution to the field and set a new standard for many years to come.

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SECTION 1

Bones of the oral-dental and
craniofacial complex

Embryology of craniofacial bones

Antonio Nanci and Pierre Moffatt

In this chapter, we provide a general overview of embryological events pertinent to the development of the bony structures of the craniofacial complex, which has been largely adapted from Ten Cate's *Oral Histology Textbook* (Nanci 2007). We also briefly review well-established molecular concepts at play in craniofacial patterning and some of the more recent developments in this field. In this context, processes have been abridged and only detailed when necessary for logical flow. For a more comprehensive treatise, readers are referred to this chapter's references.

The cranial region of early jawless vertebrates comprised (1) cartilaginous elements to protect the notochord and the nasal, optic, and otic sense organs (neurocranium); and (2) cartilaginous rods supporting the branchial (pharyngeal) arches in the oropharyngeal region (viscerocranium). Together, the neurocranium and the viscerocranium formed the chondocranium. As vertebrates evolved, they came to develop jaws through modification of the first arch cartilage, with the upper portion becoming the maxilla and the lower portion the mandible. In addition, they acquired larger sensory elements resulting in a significant expansion of the head region. Bony skeletal elements (the dermal bones), evolved for protection, formed the vault of the skull and the facial skeleton that included bony jaws and teeth. The cephalic expansion required a new source of connective tissue that was achieved by the epitheliomesenchymal transformation of cells from the neuroectoderm. Indeed, the neural origin of craniofacial bones distinguishes them from other skeletal bones, and may, in part, explain why in certain cases bones at these two sites are differentially affected (e.g., osteoporosis). Comparison

between the cranial components of the primitive vertebrate skull and the cranial skeleton of a human fetus is shown in Figure 1.1.

Head formation

Neural crest cells (NCCs) from the midbrain and the first two rhombomeres transform and migrate as two streams to provide additional embryonic connective tissue needed for craniofacial development (Figure 1.2). The first stream provides much of the ectomesenchyme associated with the face, while the second stream is targeted to the first arch where they contribute to formation of the jaws. NCCs from rhombomere 3 and beyond migrate into the arches that will give rise to pharyngeal structures. Since homeobox (Hox) genes are not expressed anterior to rhombomere 3, a different set of coded patterning genes has been adapted for the development of cephalic structures. This new set of genes, reflecting the later development of the head in evolutionary terms, includes the Msx (muscle segment Hox), Dlx (distal-less Hox), Barx (BarH-like Hox) gene families.

Branchial arches and formation of the mouth

The mesoderm in the pharyngeal wall proliferates, forming as six cylindrical thickenings known as *branchial* or *pharyngeal arches*. Four of these arches are major; the fifth and sixth arches are transient structures in humans. The arches expand from the lateral wall of the pharynx toward the midline.

The inner aspect of the branchial arches is covered by endoderm (with the exception of the ectoderm of the

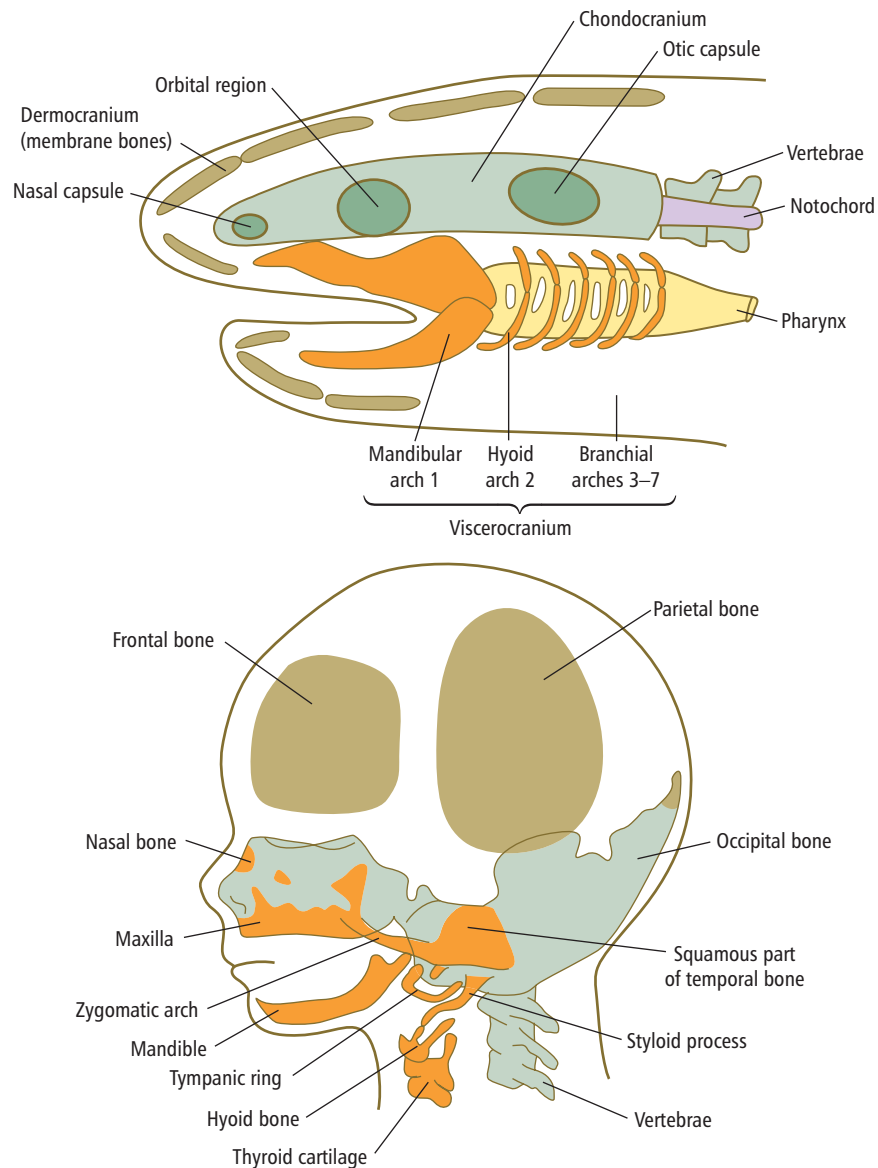


Figure 1.1 The major components of the primitive vertebrate cranial skeleton and the distribution of these same components in a human fetal head. (Adapted from Carlson 2004, with permission from Elsevier Ltd.)

first arch because it forms in front of the buccopharyngeal membrane). The central core consists of mesenchyme derived from lateral plate mesoderm that is invaded by NCCs. The resulting ectomesenchyme condenses to form a bar of cartilage, the arch cartilage. The cartilage of the first arch is called *Meckel's cartilage*, and that of the second is *Reichert's cartilage*; the remaining arch cartilages are not named.

The primitive oral cavity is at first bounded above (rostrally) by the frontal prominence, below (caudally) by the developing heart, and laterally by the first branchial arch. With the midventral expansion of arches, the cardiac plate is pushed away, and the floor of the mouth is formed by the first, second, and third branchial arches.

At about the middle of the fourth week of gestation, the first branchial arch establishes the maxillary process, so that the oral cavity is limited cranially by the frontal prominence covering the rapidly expanding forebrain, laterally by the newly formed maxillary process, and ventrally by the first arch (now called the *mandibular process*; Figure 1.3).

Formation of the face, primary palate, and odontogenic epithelium

Early development of the face is dominated by the proliferation and migration of ectomesenchyme involved in the formation of the primitive nasal cavities. At about 28

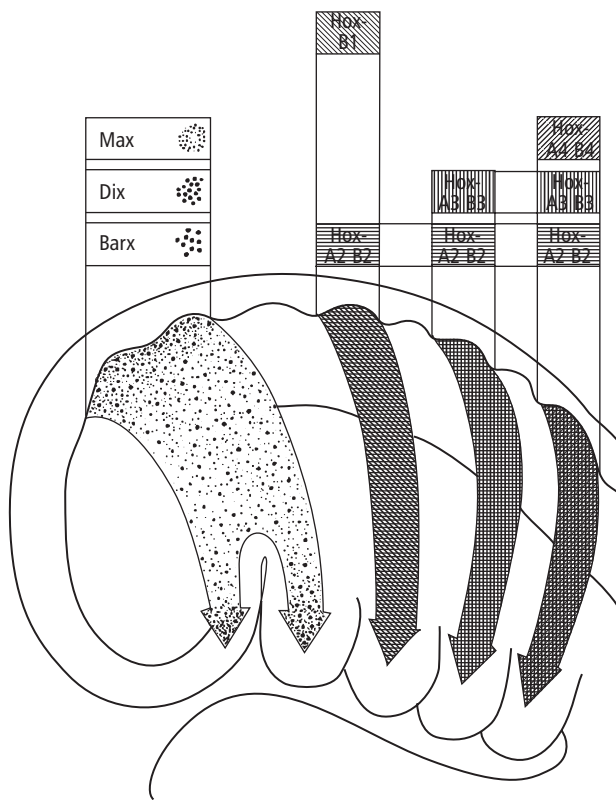


Figure 1.2 Migrating neural crest cells (NCCs) express the same homeobox (Hox) genes as their precursors in the rhombomeres from which they derive. Note that Hox genes are not expressed anterior to rhombomere 3. A new set of patterning genes (Msx, Dlx, and Barx) has evolved to bring about the development of cephalic structures so that a “Hox code” also is transferred to the branchial arches and developing face. (Reprinted from Nanci 2007, with permission from Elsevier Ltd.)

days, two localized thickenings develop within the ectoderm of the frontal prominence just rostral to the oral cavity. The mesenchyme at the periphery of these so-called olfactory placodes undergoes rapid proliferation giving rise to two horseshoe-shaped ridges on the frontal prominence. The lateral arm of the horseshoe is called the *lateral nasal process*, and the medial arm is called the *medial nasal process*. The region of the frontal prominence, where these changes take place and the nose will develop, is now referred to as the *frontonasal process*.

The maxillary process grows medially and approaches the lateral and medial nasal processes (Figure 1.4). The medial growth of the maxillary process pushes the medial nasal process toward the midline, where it merges with its anatomic counterpart from the opposite side. The medial nasal processes of both sides, together with the frontonasal process, give rise to the middle portion of the nose, the middle portion of the upper lip, the anterior portion of the maxilla, and the primary palate.

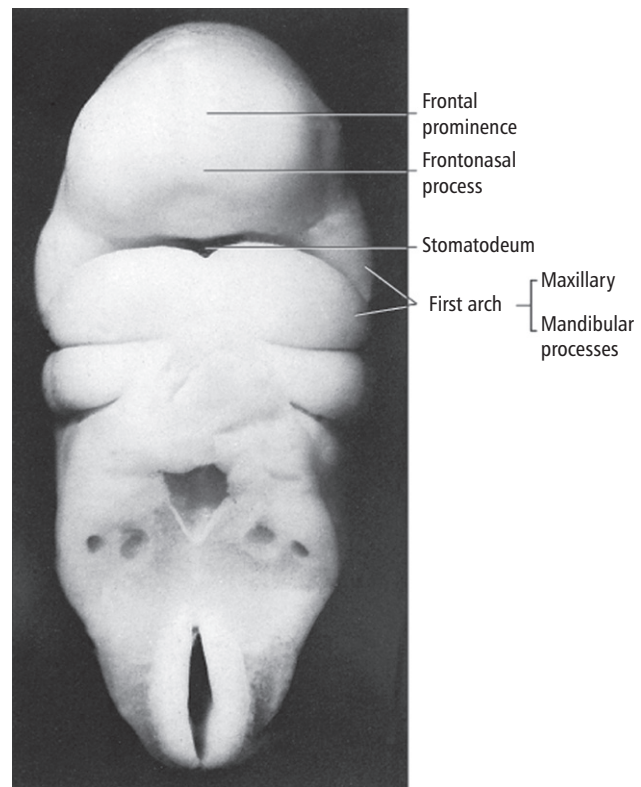


Figure 1.3 A 27-day embryo viewed from the front. The beginning elements for facial development and the boundaries of the stomatodeum are apparent. The first arch gives rise to maxillary and mandibular processes. (Reprinted from Nanci 2007, with permission from Elsevier Ltd.)

The maxillary process fuses with the lateral nasal process to form the lateral wings of the nose and cheek areas.

The face develops between the 24th and 38th days of gestation. As fusion of facial processes occurs, the epithelium on the inferior border of the maxillary and medial nasal processes and the superior border of the mandibular arch begin to proliferate and thicken. These thickened areas will soon give rise to an arch-shaped continuous plate of odontogenic epithelium on both the maxilla and the mandible.

Formation of the secondary palate

Initially, there is a common oronasal cavity bounded anteriorly by the primary palate. The subsequent development of the secondary palate creates a distinction between the oral and nasal cavities. Its formation commences between seven and eight weeks and completes around the third month of gestation. Three outgrowths appear in the oral cavity: the nasal septum grows downward from the frontonasal process along the midline, and two palatine shelves, one from each side, extend from the maxillary processes toward the midline. The

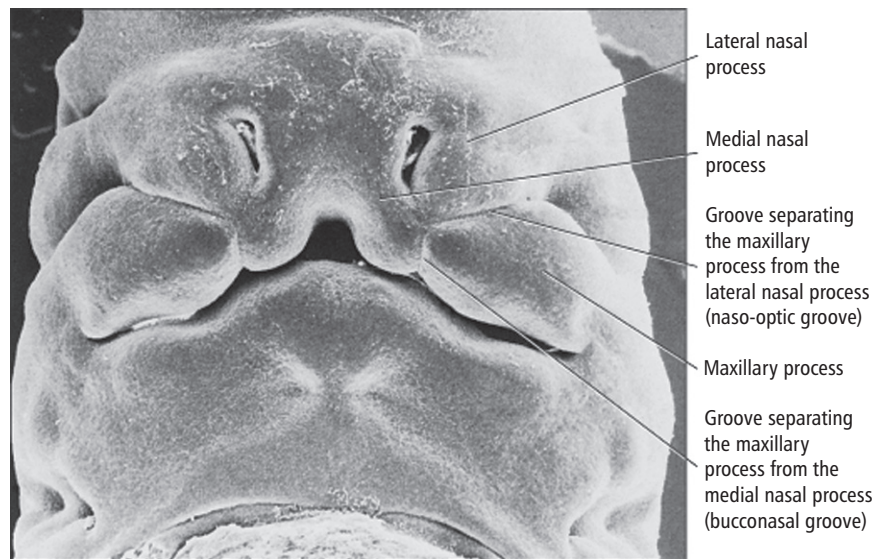


Figure 1.4 Scanning electron micrograph (SEM) of a human embryo at around six weeks. (Reprinted from Nanci 2007, with permission from Elsevier Ltd.)

septum and the two shelves converge and fuse along the midline, thus separating the primitive oral cavity into nasal and oral cavities. As the two palatine shelves meet, adhesion of the epithelia occurs. The epithelial cells at the seam undergo epitheliomesenchymal transformation, and they acquire mesenchymal characteristics and the ability to migrate, thus establishing continuity between the fused processes. The closure of the secondary palate proceeds gradually from the primary palate in a posterior direction.

Development of the skull

The skull can be divided into three components: the cranial vault, the cranial base, and the face (Figure 1.5). Membranous bone forms the cranial vault and face while the cranial base undergoes endochondral ossification. Some of the membrane-formed bones may develop secondary cartilages to provide rapid growth.

Intramembranous bone formation was first recognized when early anatomists observed that the fontanelles of fetal and newborn skulls were filled with a connective tissue membrane that was gradually replaced by bone during the development and growth of the skull. During this process, ectomesenchymal cells proliferate and condense at multiple sites within each bone of the cranial vault, maxilla, and body of the mandible. At these sites of condensed mesenchyme, osteoblasts differentiate and begin to produce bone. This first embryonic bone forms rapidly and is termed *woven bone*. At first, the woven bone takes the form of spicules and trabeculae, but progressively these forms fuse into thin bony plates

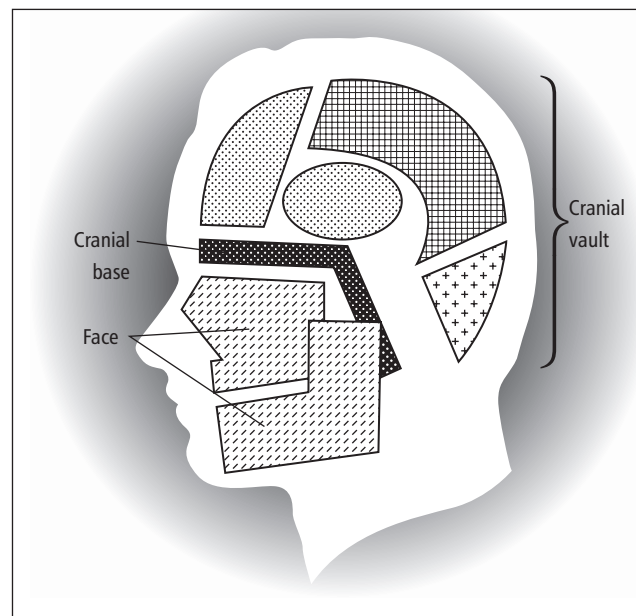


Figure 1.5 Subdivisions of the skull. (Reprinted from Nanci 2007, with permission from Elsevier Ltd.)

that may combine to form a single bone. In general, there is resorption on endosteal surfaces and bone formation on periosteal ones. However, depending on adjacent soft tissues and their growth, segments of the periosteal surface of an individual bone may contain focal sites of bone resorption. For instance, growth of the tongue, brain, and nasal cavity and lengthening of the mandible body require focal resorption along the periosteal surface.

Conversely, segments of the endosteum of the same bone simultaneously may become a forming surface, resulting in bone drift. Woven bone of the early embryo and fetus turns over rapidly. There is a rapid transition from woven bone to lamellar bone during late fetal development and the first years of life.

As fetal bones begin to assume their adult shape, continued proliferation of soft connective tissue between adjoining bones brings about the formation of sutures and fontanelles. Sutures play an important role in the growing face and skull. Found exclusively in the skull, sutures are the fibrous joints between bones. However, sutures allow only limited movement. Their function is to permit the skull and face to accommodate growing organs such as the eyes and brain.

The periosteum of a bone consists of two layers: an outer fibrous layer and an inner cellular or osteogenic layer apposed to the surface of the bone. At sutures, the outer fibrous layers of the two adjacent bones involved in the joint extend and fuse across the gap between the bones. The osteogenic layer and part of the fibrous layer of each bone run down through the gap between the bones. When these are forced apart, for example by the growing brain, the structural arrangement at the suture allows bone formation at the margins while keeping the bones separated yet strongly tied together.

Endochondral bone formation occurs at the articular extremity of the mandible and base of the skull. Early in embryonic development, a condensation of ectomesenchymal cells occurs. Cartilage cells differentiate from these cells, and a perichondrium forms around the periphery, giving rise to a cartilage model that eventually is replaced by bone.

Development of the mandible and maxilla

As indicated above, the mandible and the maxilla form from the tissues of the first branchial arch, the mandible forming within the mandibular process and the maxilla within the maxillary process that outgrows from it.

Mandible

The cartilage of the first arch (Meckel's cartilage) forms the lower jaw in primitive vertebrates. In human beings, Meckel's cartilage has a close positional relationship to the developing mandible but is believed to make no direct contribution to it. At six weeks of development, this cartilage extends as a solid hyaline cartilaginous rod surrounded by a fibrocellular capsule from the developing ear region (otic capsule) to the midline of the fused mandibular processes (Figure 1.6). The two cartilages of each side do not meet at the midline but are separated by a thin band of mesenchyme.

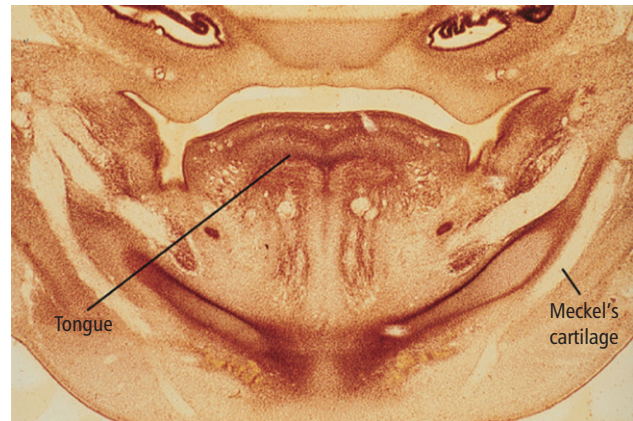


Figure 1.6 Slightly oblique coronal section of an embryo demonstrating almost the entire extent of Meckel's cartilage. (Reprinted from Nanci 2007, with permission from Elsevier Ltd.)

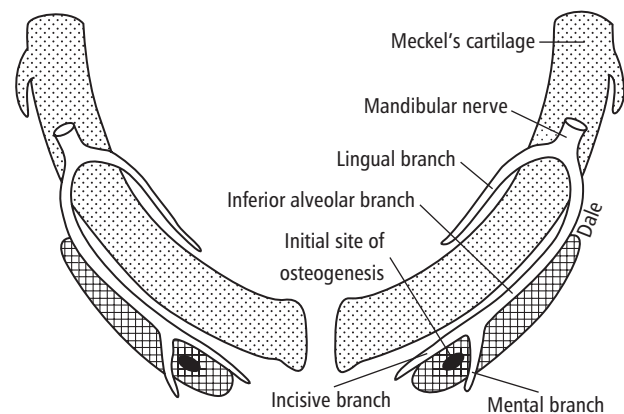


Figure 1.7 Site of initial osteogenesis related to mandible formation. Bone formation extends from this anteriorly and posteriorly along Meckel's cartilage. (Reprinted from Nanci 2007, with permission from Elsevier Ltd.)

On the lateral aspect of Meckel's cartilage, during the sixth week of embryonic development, a condensation of ectomesenchyme occurs in the angle formed by the division of the inferior alveolar nerve and its incisor and mental branches. At seven weeks, intramembranous ossification begins in this condensation, forming the first bone of the mandible (Figure 1.7). From this center of ossification, bone formation spreads rapidly anteriorly to the midline and posteriorly toward the point where the mandibular nerve divides into its lingual and inferior alveolar branches. This spread of new bone formation occurs anteriorly along the lateral aspect of Meckel's cartilage, forming a trough that consists of lateral and medial plates that unite beneath the incisor nerve. This trough of bone extends to the midline, where it comes into approximation with a similar trough formed in

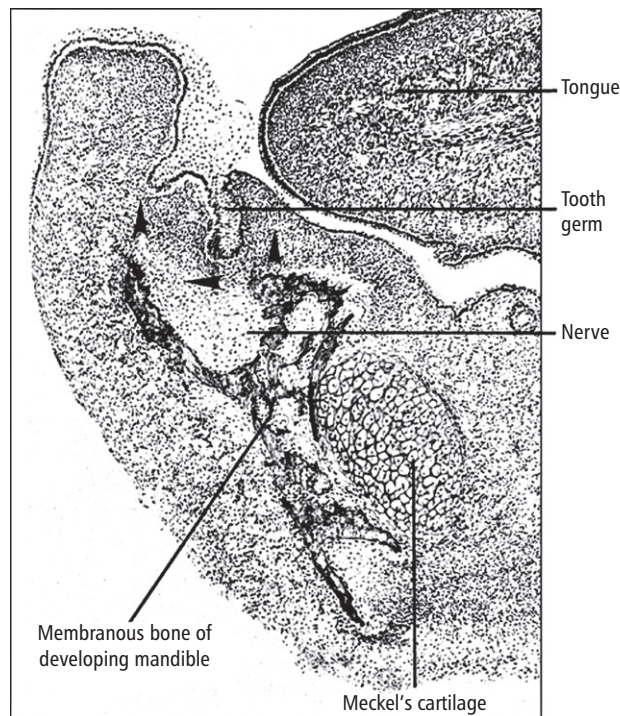


Figure 1.8 Photomicrograph of a coronal section through an embryo showing the general pattern of intramembranous bone deposition associated with formation of the mandible. The relationship among nerve, cartilage, and tooth germ is evident. Arrowheads indicate the future directions of bone growth to form the neural canal and lateral and medial alveolar plates. (Reprinted from Nanci 2007, with permission from Elsevier Ltd.)

the adjoining mandibular process (Figure 1.8). The two separate centers of ossification remain separated at the mandibular symphysis until shortly after birth.

Similarly, a backward extension of ossification along the lateral aspect of Meckel's cartilage forms a gutter that is later converted into a canal that contains the inferior alveolar nerve. This backward extension of ossification proceeds in the condensed mesenchyme to the point where the mandibular nerve divides into the inferior alveolar and lingual nerves. From this bony canal, medial and lateral alveolar plates of bone develop in relation to the forming tooth germs so that the tooth germs occupy a secondary trough of bone. This trough is partitioned, and thus the teeth come to occupy individual compartments that are finally enclosed totally by growth of bone over the tooth germ (Figure 1.8). The ramus of the mandible develops by a rapid spread of ossification posteriorly into the mesenchyme of the first arch, turning away from Meckel's cartilage. Thus, by 10 weeks the rudimentary mandible is formed almost entirely by membranous ossification, with no apparent involvement of Meckel's cartilage.

The further growth of the mandible until birth is influenced strongly by the appearance of three secondary cartilages and the development of muscular attachments: (1) the condylar cartilage, which is most important; (2) the coronoid cartilage; and (3) the symphyseal cartilage.

The condylar cartilage appears during the 12th week of development and rapidly forms a cone-shaped or carrot-shaped mass that occupies most of the developing ramus. This mass of cartilage is converted quickly to bone by endochondral ossification so that at 20 weeks, only a thin layer of cartilage remains in the condylar head. This remnant of cartilage persists until the end of the second decade of life, providing a mechanism for growth of the mandible in the same way as the epiphyseal cartilage does in the limbs.

The coronoid cartilage appears at about four months of development, surmounting the anterior border and top of the coronoid process. Coronoid cartilage is a transient growth cartilage and disappears long before birth. The symphyseal cartilages, two in number, appear in the connective tissue between the two ends of Meckel's cartilage but are entirely independent of it. They are obliterated within the first year after birth.

Maxilla

The maxilla also develops from a center of ossification in the mesenchyme of the maxillary process of the first arch. No arch cartilage or primary cartilage exists in the maxillary process, but the center of ossification is associated closely with the cartilage of the nasal capsule. As in the mandible, the center of ossification appears in the angle between the divisions of a nerve (i.e., where the anterosuperior dental nerve is given off from the inferior orbital nerve). From this center, bone formation spreads posteriorly below the orbit toward the developing zygoma and anteriorly toward the future incisor region. Ossification also spreads superiorly to form the frontal process and downward to form the lateral alveolar plate for the maxillary tooth germs. Ossification also spreads into the palatine process to form the hard palate. The medial alveolar plate develops from the junction of the palatine process and the main body of the forming maxilla. This plate, together with its lateral counterpart, forms a trough of bone around the maxillary tooth germs that eventually become enclosed in bony crypts.

A secondary cartilage also contributes to the development of the maxilla. A zygomatic, or malar, cartilage appears in the developing zygomatic process and for a short time adds considerably to the development of the maxilla. At birth, the frontal process of the maxilla is well marked, but the body of the bone consists of little more than the alveolar process containing the tooth germs and

small though distinguishable zygomatic and palatine processes. The body of the maxilla is relatively small because the maxillary sinus has not developed. This sinus forms during the 16th week as a shallow groove on the nasal aspect of the developing maxilla.

Molecular aspects of craniofacial development: concepts and recent developments

NCC subpopulations, depending on their anteroposterior location within the neural tube, are subject to a very complex set of signaling events. A plethora of molecules is being used as cues to guide them to their ultimate destination within a restricted area of the head. The ventrolateral segmentation and migration of NCCs toward branchial arches and their eventual differentiation are tightly controlled through reciprocal signaling by neighboring cells from the endoderm and ectoderm. All molecules involved are controlled both temporally and spatially. The contribution of many of them has been deciphered with the use of genetically altered animal models (mouse, zebra fish, and chick) that often recapitulate human syndromes caused by mutations in corresponding genes.

The anteroposterior fate of NCCs is believed to be acquired before migration, but some plasticity may occur depending on environmental cues. The Hox family of transcription factors is instrumental in specifying the branchial arch. Since in evolutionary terms the head developed later, Hox genes are not expressed rostral to the first branchial arch, and the development of cephalic structures relies on a new set of coded Hox patterning genes that includes the transcription factors Otx2 (orthodenticle Hox 2), Msx, Dlx, Barx, and probably others that have not yet been fully characterized. In the second branchial arch, Hoxa2 functions to modulate the competence of NCCs toward skeletogenic signaling by fibroblast growth factors (FGFs), resulting in negative regulation of several downstream transcriptional regulators such as Pitx1 (paired-like homeodomain transcription factor 1), Lhx6 (LIM Hox protein 6), Six2 (sine oculis Hox 2), Alx4 (aristaless-like Hox 4), Bapx1 (bagpipe Hox or nk3 Hox 2), and Barx1 (BarH-like Hox 1) that are normally expressed in the first branchial arch. The mechanisms leading to the activation and repression of Hox genes in the cranial region and hindbrain are also very complex in nature, depending on epigenetic regulations and FGF8 signaling. These mechanisms provide another level of complexity, indirectly affecting transcriptional events. Notably, the remodeling machinery that modifies chromatin architecture renders DNA more or less accessible to transcription factors and co-factors. Modifier enzymes that

target nucleosomal histones (i.e., acetyltransferase and demethylase) have been described that have profound effects on craniofacial patterning.

Environmental factors that transmit repulsive and/or attractive signals are also instrumental in specifying the segregation and fate of NCCs in their migration to branchial arches. Several secreted ligands and their membrane-bound receptors provide repulsive cues, especially in the NCC-free regions of mesenchyme adjacent to rhombomeres 3 and 5. Among others, important players in this process are the membrane-anchored receptors ErbB4 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4), ephrin, and neuroligin, along with their respective soluble ligands, neuregulins, ephrins, and semaphorins. On the other hand, directional guidance (attraction) of NCCs into their respective arches is provided by another elaborate set of species-specific molecules, such as Twist, Tbx1 (T-box 1), Sdf1b/Cxcr4a (stromal cell-derived factor 1/chemokine cxc motif receptor 4), Npn1/Vegf (neuropilin 1/vascular endothelial growth factor), and Fgfr1 (fibroblast growth factor receptor 1). Intracellular-signaling cascade events and crosstalk eventually culminate in eliciting various cellular responses including proliferation, migration, differentiation, and survival or apoptosis.

Interestingly, even though both mandibular and maxillary primordia originate from similar NCCs and possess similar molecular features, they develop into very different structural entities. In the first branchial arch, a gradient of gene expression involving the Dlx family of transcription factors (1–6), the so-called intra-arch Dlx code, promotes coordinated gene expression along the dorsoventral axis that regulates jaw patterning. Distinct sets of Dlx family members are important for determining the identity of the mandible (Dlx1/2/5/6) versus the maxilla (Dlx1/2). A dramatic demonstration of the importance of the selective set of Dlx molecules in jaw specification is observed in mice lacking both Dlx5 and Dlx6 genes. Lack of Dlx5/6 causes a reversal of the mandible into a maxilla, generating an animal with two mirror-image upper jaws. Dlx5/6 activate expression of other downstream transcription factors—Dlx3/4, Hand1/2 (heart- and neural crest derivatives—expressed 1 and 2), Alx3/4, Pitx1, Gbx2 (gastrulation brain homeobox 2), and Bmp7 (bone morphogenetic protein 7)—important for mandibular development processes, and repress others, such as Pou3f3 (pou domain class 3, transcription factor 3), Foxl2 (forkhead box l2), and Irx5 (Iroquois Hox protein 5), that are themselves important for maxillary processes and under the control of Dlx1/2. Thus, Dlx family members are critical for determining the identity of the mandible versus the maxilla. Another level of complexity is brought about by

local environmental signaling crosstalk that directly or indirectly modulates the transcriptional *Dlx* program. One such regulator is endothelin, a secreted molecule produced mostly by the ectoderm that signals through the endothelin receptor *Ednra* in NCCs and promotes, possibly through *Mef2C* (mads box transcription enhancer factor 2 polypeptide c), *Dlx5/6* expression. Targeted ablation of the endothelin pathway in mice causes duplication of maxillary processes, whereas ectopic expression induces duplication of mandibular processes. Other signaling events, coming from the endoderm, such as *Vegf* and *Shh* (sonic hedgehog), or the ectoderm—*Fgf*, *Bmp*, and *Wnt* (wingless-type mouse mammary tumor virus [MMTV] integration site family)—also promote dorsoventral guidance by modulating many different processes, such as migration, survival, apoptosis, and/or differentiation.

More recently, posttranscriptional mechanisms contributing to the regulation of NCC development have been uncovered. Of mention is the effect of specific micro-RNAs (miRNAs) that control the half-life of targeted gene messenger RNAs (mRNAs) by interacting with the 3' untranslated region and thus repressing translation and/or targeting for degradation. For instance, the expression of miR-452, abundant in early NCCs, directly targets *Wnt5A*, consequently lowering the activity of downstream effector signaling molecules *Shh* and *Fgf8*, at least in the mandibular region of the first branchial arch. miR-452 is an indirect positive modulator of *Dlx2* expression, itself controlled by *Fgf8*. Another function attributed to *Wnt5a* is the activation of noncanonical *Wnt* pathways through the *Frizzled* (*Fzd*) and activating *Disheveled* (*Dsh*) proteins that regulate the orientation of cell structures through the planar cell polarity (*Pcp*) genes. The effects of *Pcp*, promoting cell-to-cell contacts, are to induce a coordinated polarized migratory path of NCCs in the branchial arch, and to induce cartilage outgrowth and chondrogenesis of cranial base structures and the nasal septum.

The species-specific patterning of the head and face, especially the shape and size of the beak and muzzle, has been suggested to depend on the canonical (beta-catenin-dependent) *Wnt* signaling pathway that seems to be an upstream modulator of critical effector molecules, such as *Fgf8*, *Bmp2*, and *Shh*, present in the frontonasal ectodermal zone (FEZ) center. FEZ is another major determinant of species-specific patterning and outgrowth of the upper face. Variation in the organization, relative size, and position of the FEZ, together with other molecules like calcium-dependent calmodulin, is partly responsible for the very different shapes encountered in nature. These data indicate the complexity of the

various pathways that contribute to facial outgrowth by regulating cell proliferation and differentiation.

Conclusions, futures orientations, and clinical perspectives

In this chapter, we have described the basic embryological events and provided an overview of major signaling interactions and molecules implicated in craniofacial development and morphogenesis. While our understanding of molecular analyses has made significant progress, the cell biological activities resulting from various molecular cascades remain largely unexplored. Planar polarity genes are attracting much attention not only because of their role in regulating cell polarity and morphogenesis, but also because of their implication in positioning cellular structures and coordinating activities such as cell intercalation. One such structure is the cilium that is found on the surface of most vertebrate cells and acts as a mechanical and chemical sensor. Ciliary dysfunction is present in some syndromes, such as facial-digital syndrome and Bardet–Biedl syndrome that exhibit facial changes, as well as cleft palate and micrognathia. Experimentally, it has been shown that a neural crest–targeted mutation of the *kif3* gene, encoding for a kinesin-like protein implicated in cilogenesis and intraflagellar transport, affects polarized growth and cell shape, resulting in shortened mandibles and defects in development of the cranial base. It is worth noting that some phenotypes resulting from ciliopathies are linked to perturbations of signaling pathways, including that of *Wnt*.

Current treatments for craniofacial malformations such as craniosynostosis (premature fusion of sutures) and cleft lip and palate are essentially surgical. Such interventions can in some cases lead to serious complications or require multiple interventions. Clearly, a better understanding and especially integration of cell, tissue, and molecular events implicated in craniofacial development and formation are necessary for the rational design of genetic and pharmacological strategies for correcting malformations. While interventions after birth with novel therapeutic approaches would represent a major improvement, the eventual ability to intervene in utero would allow correcting problems early on so that subsequent development could follow its normal course. In utero interventions to correct aberrant signaling could exploit recent developments in gene therapy and the stem and progenitor potential of NCCs. The feasibility of successful prenatal manipulations, however, still remains in the realm of wishes for now because of the extremely early (first weeks of gestation) and narrow

window in which potential intervention would need to be performed.

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Clinical correlate: cleft lip and palate

Emily R. Gallagher and Joel Berg

Development of the face and mouth occurs primarily between the fourth and 10th weeks post conception. The craniofacial region arises from neural crest cells, which form the frontonasal prominence and the paired maxillary and mandibular processes by the end of the fourth week. The medial nasal processes and the maxillary prominences fuse by the end of the sixth week, resulting in the formation of the upper lip and the primary palate. Also during the sixth week of development, the secondary palate forms from the paired palatal shelves, which are outgrowths from the maxillary processes. These grow vertically at first and then fuse to form a horizontal position above the tongue. This tissue differentiates into bone and muscle that form the hard and soft palate, respectively. The palate fuses longitudinally along the midline but also anteriorly with the primary palate and nasal septum. These tissues are normally fused by the 10th week after conception (Sperber 2002; for review, see Chapter 1, this volume). The successful development of a palate, which divides the floor of the mouth from the nasal septum, allows for simultaneous feeding and respiration. Unsuccessful fusion will result in a unilateral or bilateral cleft of the lip and/or palate (Figure 2.1).

Epidemiologic studies suggest that oral clefts occur in about 1 in 700 live births (Mossey & Castillia 2003). The prevalence of cleft lip and palate varies among ethnic groups, with Native Americans and Asians having a higher prevalence, but the prevalence of cleft palate alone is not related to ethnic background (Saal 2002). Cleft lip and palate is more frequent in males, while cleft palate alone is more prevalent in females (Mossey *et al.* 2009). Large studies of individuals in Europe with orofacial clefting found that 55% of the cases of cleft palate

alone were isolated, 18% had other anomalies, and 27% had known syndromes (Calzolari *et al.* 2004). In patients with cleft lip and palate, 71% of cases were isolated and 29% were associated with other anomalies (Calzolari *et al.* 2007). Hundreds of syndromes are known to be associated with orofacial clefts. Among the most common are Van der Woude's syndrome, an autosomal dominant disorder with clefting and lower lip pits; velocardiofacial syndrome, which is caused by a small deletion in 22q11.2; and Pierre Robin sequence, which is characterized by the triad of micrognathia, glossoptosis, and a U-shaped cleft palate.

Various genes are involved in regulating facial development, including transcription regulators, growth factors, and signaling molecules. Much of our understanding of the complex regulation among these genes comes from studying animal models (*Drosophila*, mice, and chicks). A change in the expression or structure of these genes may result in the formation of an orofacial cleft.

Various environmental factors thought to be involved in clefting have been studied, including maternal nutritional status and in utero exposure to tobacco, alcohol, and medications. A large study using birth defects registries found an increased odds ratio (OR) of clefting associated with maternal smoking. There was a stronger association with bilateral clefts (OR 1.7, 95% CI 1.2–2.6) than with unilateral clefts (OR 1.3, 95% CI 1.0–1.6). The effect was stronger with heavy smoking, but there was not an increased association of maternal smoking with cleft palate alone (Honein *et al.* 2007). The association of alcohol and clefting is not as well understood, though exposure to alcohol in utero is believed to be associated

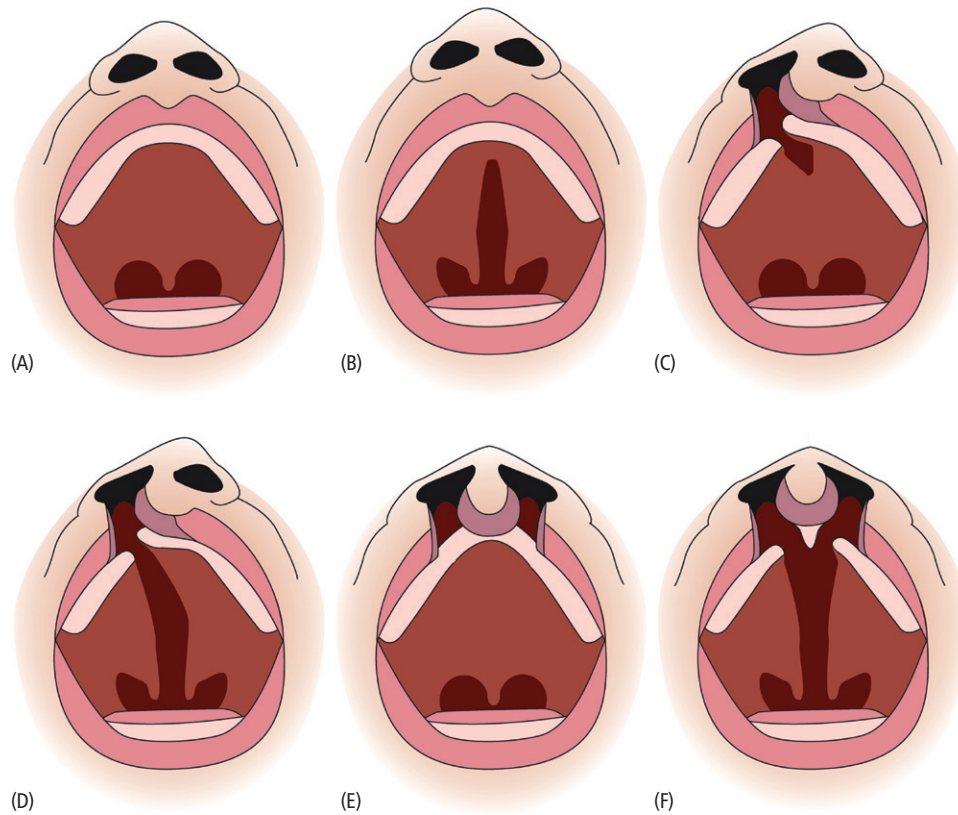


Figure 2.1 Types of orofacial clefts. A. Intact lip and palate. B. Cleft palate. C. Incomplete unilateral cleft of the lip and alveolar ridge. D. Incomplete bilateral cleft lip. E. Complete unilateral cleft lip and palate. F. Complete bilateral cleft lip and palate.

with an increased risk of clefting. Taking prenatal vitamins has been associated with a decreased risk of orofacial clefting, possibly due to folic acid (Wehby & Murray 2010). A meta-analysis found a protective effect associated with maternal multivitamin use for cleft lip and palate (OR 0.75, 95% CI 0.65–0.88), but the effect was not significant for cleft palate alone (OR 0.88, 95% CI 0.76–1.01; Johnson & Little 2008). Other studies have suggested that fetal exposure to drugs with retinoic acid, anticonvulsants, and corticosteroids could be associated with an increased risk of oral clefting. However, many of these studies are flawed by sample size, confounding, or publication bias (Mossey *et al.* 2009).

Transabdominal ultrasound during pregnancy is often able to detect cleft lip and palate. A systematic review found variable results in the ability of two-dimensional (2D) ultrasounds to correctly diagnose cleft lip and palate, but three-dimensional (3D) ultrasounds were reliably able to detect clefts (Maarse *et al.* 2010). The severity of the cleft lip may not be accurately predicted by ultrasound, and the ability of ultrasound to diagnose a cleft palate remains poor. Learning about an orofacial cleft during pregnancy allows the family time to plan for the child's needs at the time of birth, to meet a team that

provides cleft care, and to be prepared in the delivery room for a baby with a cleft.

Case presentation

The patient is a four-year-old Caucasian male who has had a typical course for an isolated unilateral cleft lip and palate. He presents today for routine dental evaluation and oral hygiene assessment. Photographs were taken to document the current development of the primary dentition. The appearance of the primary tooth maxillary arch is typical, with the maxillary left primary lateral incisor rotated within the area of the cleft and the adjacent primary canine positioned lingual to the central portion of the alveolar ridge. There is a noted absence of alveolar ridge height in the cleft that will be corrected at the time of the alveolar ridge bone grafting around eight years of age.

The patient was diagnosed prenatally, allowing his family time to meet a craniofacial team and plan for the patient's feeding needs. Babies with palatal clefts are not able to produce suction. They are, therefore, rarely able to breastfeed or use a regular bottle. Alternatives for feeding include options whereby parents can help trans-

fer milk by squeezing a bottle while the baby simulates a suck. Feeding should be closely monitored during infancy to ensure that the baby is growing well and will be healthy for surgeries during the first year. The patient was evaluated by the craniofacial team in the first week of life and subsequently was given a Haberman bottle for feeding. He maintained normal growth throughout infancy. He began eating solid food at six months of age and suffered mild nasal regurgitation but no other difficulties.

In addition to seeing a pediatrician on a craniofacial team soon after birth and a nurse with expertise in feeding infants with clefts, the patient also saw a plastic surgeon. Depending on the severity of the cleft, the surgeon may recommend presurgical orthodontics such as nasoalveolar molding (NAM) or a Latham device. In some cases, the surgeon may recommend taping across the lip to use tension to bring the edges of the cleft closer together. This makes the surgical repair easier and provides a better surgical outcome.

The patient's cleft lip was repaired at three months of age, and his palate was repaired at 12 months of age, typical timing for both procedures. Throughout his first year, feeding, growth, and development were monitored closely. If there had been concern for other anomalies or an associated syndrome, further diagnostic evaluation or referral to a geneticist would have been recommended.

Hearing was also monitored closely, particularly during the first year of life. A cleft palate is often associated with Eustachian tube dysfunction and chronic middle ear effusion. The patient had mild hearing loss bilaterally when his hearing was checked at nine months of age. He subsequently had ear tubes placed by an otolaryngologist at the time of his palate repair.

A cleft palate interferes with speech development, even after the palate is surgically repaired. The patient's speech development was assessed when he was two years old, and he was found to have velopharyngeal insufficiency (VPI), an inability to create adequate intraoral pressure. He had speech therapy for the next 18 months but continued to have VPI. This was corrected with a Furlow palatoplasty, a procedure to lengthen the palate and improve his ability to close the distance between the soft palate and posterior pharyngeal wall. A subsequent speech assessment showed resolution of his VPI.

The patient has had routine dental care with no dental caries lesions present. While he will have an alveolar bone graft when his permanent teeth begin to erupt, maintaining healthy primary teeth is important for bone health adjacent to the alveolar cleft. Examination of the mouth today reveals exceptionally clean teeth, providing a low risk of experiencing caries lesions in the future. There may be an increased prevalence of dental caries

associated with cleft lip and palate, possibly related to poor enamel on the teeth adjacent to the cleft, although the data are somewhat inconclusive (Hasslof & Twetman 2007). Keeping the teeth plaque free is essential in tooth decay prevention. As with any child under the age of eight years, the parents or caregivers must assist the child in tooth brushing and flossing, as most children do not have the needed dexterity to independently brush their teeth well until eight or nine years of age.

Throughout his teenage years, this patient will continue to have periodic visits with the craniofacial team to discuss concerns related to peer acceptance around differences in speech and facial appearance, to continue assessment of his development and overall health, and to monitor midfacial growth. If he has significant midface hypoplasia, he may need to undergo surgery to advance his midface when his skeletal growth is complete (Figures 2.2, 2.3, and 2.4).



Figure 2.2 Occlusal view of the maxillary arch.



Figure 2.3 Facial view of the maxillary arch, left side.



Figure 2.4 Facial view of patient showing excellent surgical results with minimal evidence of left-side cleft lip.

Summary

Orofacial clefting, which includes cleft lip, cleft lip and palate, and cleft palate alone, is a common birth defect, affecting about 1:700 live births. Clefts can be isolated and not associated with other defects or related to an underlying syndrome. The etiology of clefting is unclear, but it is thought to involve complex genetic and environmental interactions. The primary disruptions associated with clefts include difficulty with feeding during infancy, hearing loss associated with chronic middle ear effusion, speech disorders, and facial and dental differences.

Children with orofacial clefts require management by a multidisciplinary team for optimal outcomes. Composition of the team should include individuals from audiology, nursing, nutrition, oral and maxillofacial surgery, orthodontics, otolaryngology, pediatrics, pediatric dentistry, plastic surgery, social work, and speech–language pathology (American Cleft Palate–Craniofacial Association 2007). In addition to achieving the desired outcomes by adulthood, team care provides the most efficacious and cost-effective way of managing these patients and results in optimal outcomes (Vargervik *et al.* 2009).

The patient is a four-year-old Caucasian male who was diagnosed prenatally with an isolated cleft lip and palate. He had his lip and palate repaired during his first year of life and continued to work with the Craniofacial Center at Seattle Children's Hospital for ongoing needs related to his speech, his mild hearing loss, and timing for future alveolar bone grafting and orthodontics.

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3

Cell and molecular biology of the osteoclast and bone resorption

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The maintenance of normal bone mass during adult life depends on the balanced action of bone-resorbing osteoclasts (OCs) and bone-forming osteoblasts (OBs). This coordinated process continuously renews mineralized tissue throughout the skeleton to maintain an optimum bone structure adapted to mechanical and metabolic demands. Excessive resorption leads to pathological bone loss, as it occurs in osteoporosis, Paget's bone disease, tumor osteolysis, rheumatoid arthritis, and, most importantly for this chapter, periodontitis. Research on the bone destruction associated with periodontal disease has highlighted the importance of a tight and local control of OC differentiation and function (Han *et al.* 2007). The overall rate of osteoclastic bone resorption is regulated at two main levels: (1) determining the number of OCs through the regulation of the OC precursor pool and their rate of differentiation (i.e., regulating osteoclastogenesis), and (2) determining the bone-resorbing activity of individual OCs through the regulation of their key functional features.

Regulation of osteoclast number

Origin of osteoclasts

It is well established that OCs originate from hematopoietic stem cell-derived progenitors with myeloid-restricted differentiation potential (Teitelbaum 2000). These progenitors reside in the bone marrow where they differentiate into monocytes that eventually exit to the blood and circulate. Monocytes then enter connective tissues and further differentiate into mononucleated OC

precursors. Mature OCs are multinucleated cells that result from the fusion of the mononucleated precursors. The hematopoietic origin of the OC precursors allows the *in vitro* generation of OCs from a variety of tissues including bone marrow, circulating blood, spleen, and embryonic liver. Various subgroups of monocytes, distinguished by the expression of cell surface proteins, circulate in the blood. It is well established that these subgroups do not have the same ability to generate OCs. This raises the possibility that OC precursors originate from a specific subset of monocytes (Lorenzo *et al.* 2008).

The myeloid progenitors that will eventually differentiate into OCs also give rise to macrophages and dendritic cells. These different cell types share signaling pathways and transcription factors that direct their differentiation (e.g., PU.1, discussed in this chapter). However, mature OCs are unique in their capacity to efficiently resorb bone. OCs also express high levels of relatively specific markers—tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK), integrin $\alpha_v\beta_3$, and calcitonin receptor—that are mostly absent from macrophages and dendritic cells.

Recent studies suggest that plasticity exists between the different cell types originating from myeloid precursors and that mature cells can transdifferentiate into another cell type. Thus, OCs can be generated from dendritic cells *in vitro* in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL), as well as from pro-B lymphocytes and macrophages. The importance of such mechanisms *in vivo* is still unclear but could be

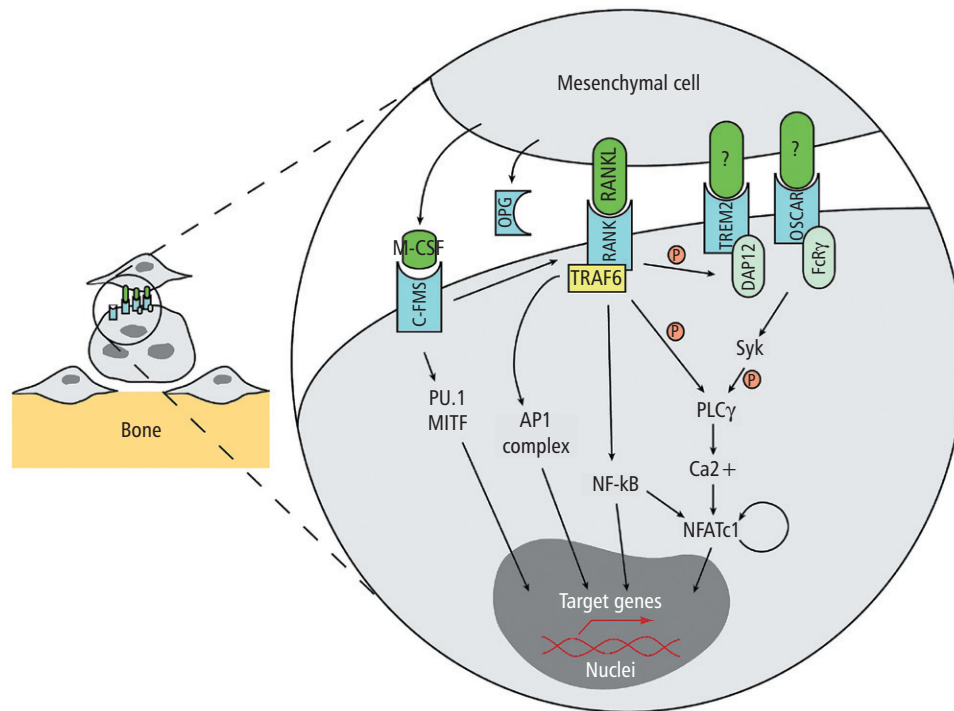


Figure 3.1 Principal signaling pathways from c-Fms, RANK, and the co-stimulatory receptors TREM2 and OSCAR involved in osteoclastogenesis. The ligands of these receptors are secreted by mesenchymal cells or present on their membrane. The ligands for the co-stimulatory receptors are unknown.

of importance in diseases where increased bone resorption is associated with a deregulated immune response (e.g., periodontitis and rheumatoid arthritis).

From the hematopoietic cell to the osteoclast precursor

Two molecules (among others) expressed by bone marrow stromal cells and OBs are both required and sufficient for the differentiation of monocytic precursors into OCs: M-CSF and RANKL (Figure 3.1).

The first step in osteoclastogenesis is the commitment of the hematopoietic stem cell to the myeloid lineage. The transcription factor PU.1 plays a central role in this step. Its absence *in vivo* results in general myeloid lineage deficiencies, including macrophages and OCs. Mice that lack PU.1 develop osteopetrosis (Tondravi *et al.* 1997), a condition defined by an increased bone mass due to defective bone resorption. Another transcription factor that is crucial at this step is microphthalmia-associated transcription factor (MITF). Mice carrying a mutation in the MITF gene are osteopetrotic due to a lack of OCs (Hodgkinson *et al.* 1993). Transcription factors essential for OC precursor differentiation, proliferation, and survival are closely related to M-CSF signaling. PU.1 promotes the expression of the M-CSF receptor c-Fms and prepares the cell to respond to M-CSF. MITF is one of the nuclear targets of M-CSF in the OC. The deletion or

the silencing mutation of the genes encoding either c-Fms (Dai *et al.* 2002) or M-CSF (Yoshida *et al.* 1990) leads to the absence of macrophages and OCs and to osteopetrosis. This is exemplified by the *op/op* mouse that lacks functional M-CSF and has OC-deficient osteopetrosis due to a mutation in the gene coding for M-CSF. Interestingly, the osteopetrosis in c-Fms knockout mice is slightly worse than in the *op/op* mice, suggesting that other ligands, possibly IL-34, bind to c-Fms and partially compensate for M-CSF during osteoclastogenesis.

M-CSF expressed by stromal cells and osteoblasts activates c-Fms on osteoclast precursors. This induces the proliferation of the precursors and favors their survival by activating the extracellular signal regulated kinase (ERK) cascade via growth factor receptor-bound protein 2 (Grb-2) and Akt kinase via phosphatidylinositol 3-kinase (PI3K; Blair *et al.* 2005). Most importantly, M-CSF also induces the expression of RANK on OC precursors. The expression of RANK and the signaling events triggered by the binding of RANKL induces the transition from the myeloid progenitor to the OC precursor.

From the osteoclast precursor to the mature osteoclast

The understanding of OC differentiation has greatly evolved since the discovery of the RANK/RANKL/OPG

system. RANKL was first identified in dendritic cells and T cells along with its receptor named RANK (Anderson *et al.* 1997). The endogenous inhibitor of RANKL, the decoy receptor osteoprotegerin (OPG), was discovered after the observation that its overexpression in mice results in an OC-poor osteopetrosis (Simonet *et al.* 1997). The ratio of RANKL and OPG expressed by stromal and osteoblastic cells controls the amount of osteoclast differentiation. Several molecules known to affect osteoclast differentiation and activity, such as parathyroid hormone, vitamin D, and prostaglandin E₂, do so by modulating RANKL and OPG expression by osteoblasts. This expression varies during OB differentiation, and for this reason perturbation of OB differentiation can affect bone resorption.

When activated, RANK recruits TRAF6, which in turn activates NF- κ B, Akt, and the MAP kinase pathways, including c-jun N-terminal kinase (JNK) and p38. NF- κ B is a family of dimeric transcription factors. Among the NF- κ B members, p50 and p52 are crucial players in osteoclastogenesis, and mice that lack both p50 and p52 develop severe osteopetrosis (Iotsova *et al.* 1997). Macrophages are abundant in the p50^{-/-} p52^{-/-} mice, indicating that NF- κ B functions later than PU.1 during osteoclastogenesis. NF- κ B activity is regulated by the I κ B family of inhibitors that retain NF- κ B dimers in the cytosol. NF- κ B-activating cytokines, such as RANKL, TNF α , and IL-1, rapidly initiate the classical NF- κ B pathway through the degradation of I κ B, releasing active NF- κ B into the nucleus. The overexpression of I κ B blocks this classical pathway and inhibits osteoclastogenesis.

RANKL also induces the expression of the AP-1 transcription factor c-Fos (Karsenty & Wagner 2002). Mice without c-Fos lack OCs and are osteopetrotic but have an increased number of macrophages. Mice that overexpress c-Fos under the control of the TRAP promoter have an increased number of OCs, thus indicating that c-Fos is required for OC differentiation.

NF- κ B and c-Fos together induce the expression of a third transcription factor (i.e., a nuclear factor of activated T cells cytoplasmic 1 (NFATc1, also known as NFAT2)). This in turn induces transcription of numerous genes essential for OC precursor fusion and mature OC activity, including dendritic cell-specific transmembrane protein (DC-STAMP), TRAP, calcitonin receptor, CTSK, β_3 integrin, and osteoclast-associated receptor (OSCAR), as well as its own expression. Overexpressing NFATc1 induces the differentiation of OCs in the absence of RANKL, and several experimental approaches have shown in vivo the importance of NFATc1 for osteoclastogenesis (Negishi-Koga & Takayanagi 2009).

Both NF- κ B and AP-1 pathways are also activated by cytokines other than RANKL that are not capable alone

of inducing OC differentiation (e.g., IL-1). However, RANKL seems to be uniquely able to induce the sustained expression of NFATc1 in the OC due to RANKL's ability to induce intracellular [Ca²⁺] oscillations. This in turn induces dephosphorylation and nuclear translocation of NFATc1 (Takayanagi *et al.* 2002).

Initiation of RANKL stimulation precedes the changes in Ca²⁺ by about 24 hours, suggesting that it is not a direct consequence of RANK signaling, but rather depends on activities of proteins that are expressed downstream of RANK activation. Accordingly, several studies revealed that RANKL-induced osteoclastogenesis requires the presence of two adaptor proteins that harbor immune receptor tyrosine-based activation motifs (ITAMs), FcR γ and DAP12. These proteins activate phospholipase C γ and thereby increase intracellular Ca²⁺ signaling. The ITAM proteins are complexed to co-stimulatory receptors. FcR γ associates with OSCAR and paired immunoglobulin-like receptor A (PIR-A), while there are several DAP12-associated receptors, including TREM2 (triggering receptor expressed on monocyte 2) and SIRP β 1 (signal regulatory protein β 1; Koga *et al.* 2004). The specific ligands of the co-stimulatory receptors remain to be identified and could be expressed by OB or stromal cells or by the OC precursors themselves (Negishi-Koga & Takayanagi 2009).

In humans, mutations in DAP12 or TREM2 genes lead to Nasu-Hakola disease, which includes osteopetrotic features. The osteoclastogenic potential of peripheral mononuclear cells from these patients is greatly reduced. In DAP12^{-/-} mice, RANKL-induced OC differentiation is reduced and the mice are osteopetrotic. In contrast, the deletion of FcR γ does not induce any obvious bone phenotype, but the combined deletion of DAP12 and FcR γ worsens the osteopetrosis of the DAP12 knockout. This shows that the two adaptors can compensate for each other. Both in vivo and in vitro osteoclastogenesis are almost completely blocked in the double mutant.

Phosphorylation of the ITAM adaptors is dependent on both RANK and the co-stimulatory receptors and leads to the recruitment and activation of spleen tyrosine kinase (Syk). Syk has a critical role in OC differentiation and activity where it is activated by binding to ITAMs after their phosphorylation. When Syk and adaptors and kinases like Bruton's tyrosine kinase (Btk) and Tec are activated together by RANK, it triggers phospholipase C γ (PLC γ). PLCs are enzymes that cleave phosphatidylinositol-bis-phosphate to form the second messengers, inositol-tris-phosphate (IP3) and diacylglycerol. IP3 directly increases intracellular calcium levels by inducing the release of endoplasmic reticulum calcium stores. In the case of RANKL stimulation, PLC γ 2 is activated via DAP12 co-stimulatory signals in a Src

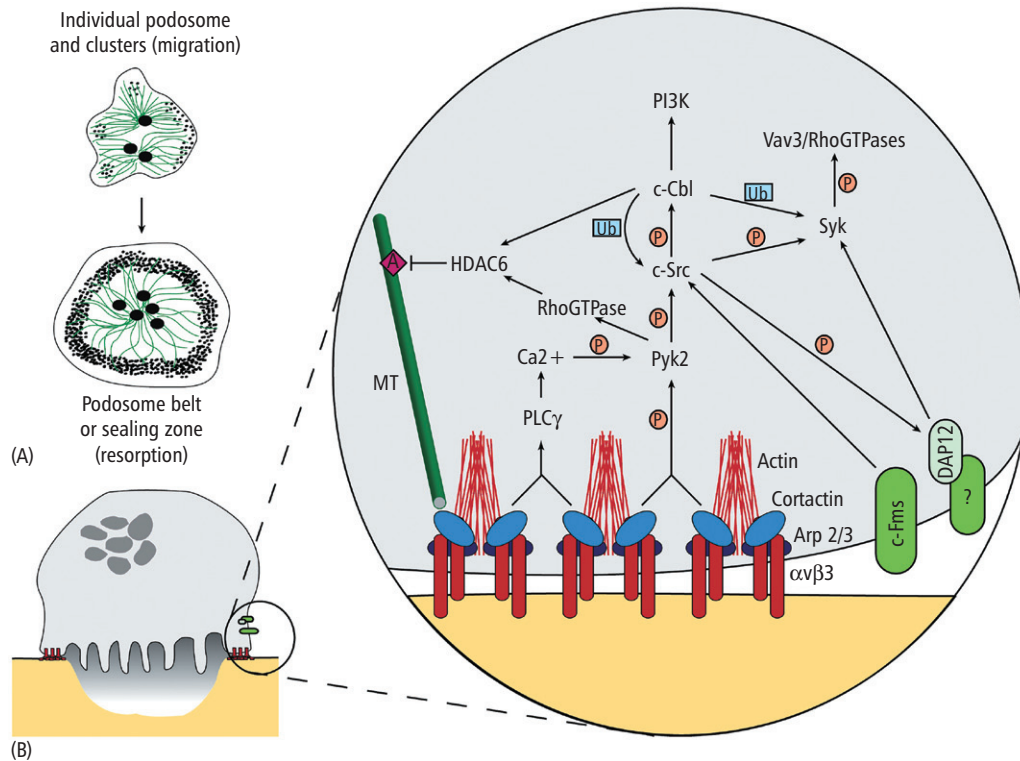


Figure 3.2 A. Podosomes are organized in different patterns during migration and resorption. This patterning is regulated by microtubules. B. Principal signaling pathways from the integrin $\alpha_v\beta_3$, c-Fms, and DAP12-associated co-stimulatory receptors involved in inducing osteoclast activity.

family kinase (SFK)–dependent manner and upregulates NFATc1 (Mao *et al.* 2006). Accordingly, PLC γ 2 knockout mice are osteopetrotic due to defective OC differentiation.

Regulation of osteoclast activity

As already mentioned, the overall rate of bone resorption by OCs is regulated at two main levels: by affecting the number of cells and by modulating their activity. The activity itself is regulated at various levels through the modulation of the key functional features of the OC. The four main functional features that define the activity of an OC are (1) its migration and adhesion to the bone surface, (2) its ability to coordinate the transport of vesicles to and from the resorption compartment, (3) its ability to acidify the resorption compartment, and (4) its ability to degrade bone matrix with secreted enzymes.

Migration and adhesion

OCs are highly mobile cells that alternate between migratory and bone-resorbing stages. Both stages are highly dependent on the interactions of the cell with the bone surface. When resorbing bone, OCs become polarized and reorganize their membranes into three distinct

domains: the sealing zone (SZ), the ruffled border (RB), and the basolateral domain (BD). Osteoclast's BD faces away from the bone surface and shares features with basolateral domains of other polarized cells. Some authors distinguish a functionally distinct part of the BD as the *functional secretory* domain, where the products of transcytosis are released. This term is actually confusing, since OC secretory activity consists mostly of polarized transport of secretory lysosomes and is directed apically toward the RB. Therefore, the term *transcytosis domain* more accurately reflects the functional properties of this region of the BD.

The first step in polarization involves a deep cytoskeletal reorganization and the formation of the actin-rich SZ (Figure 3.2). This hallmark of bone-resorbing OCs creates a tightly sealed resorption compartment between the cell and the bone surface into which protons and enzymes are secreted. The SZ consists of a dense array of interconnected podosomes (Luxenburg *et al.* 2006) that are specialized actin-rich attachment complexes used by OCs, dendritic cells, macrophages, and other cells from the monocytic lineage to adhere and migrate (Albiges-Rizo *et al.* 2009). Podosomes are highly dynamic structures that rapidly appear and disappear, undergoing fusion, fission, or sliding during their short life, which is

usually 2–4 minutes. They are distinguished from focal adhesion complexes by their geometry, their short life span, and the presence of constantly polymerizing F-actin within the complex. Podosomes comprise an actin core containing the actin filament-branching machinery, including Wiskott-Aldrich syndrome protein (WASP), neuronal WASP (N-WASP), WASP-interacting protein (WIP), the Arp2/3 complex, and cortactin, surrounded by a multimeric regulatory protein complex. This protein complex consists of integrins and integrin-associated proteins such as talin, vinculin, adaptors (Cbl, paxillin), kinases (Src, Pyk2), and Rho GTPases.

When differentiated on glass, OCs podosomes are successively organized in clusters (or patches) that evolve into several short-lived dynamic rings. These small rings then merge to form a stable peripheral belt of podosomes (also referred to as *podosome ring* or *actin ring* in the literature; Jurdic *et al.* 2006), a structure that is unique to OCs. The transition from clusters of podosomes to a peripheral belt is reversible, allowing the OC to alternate between migration and resorption stages (Figure 3.2). This transition requires the assembly and disassembly of podosomes and of the integrin-associated complex of signaling and cytoskeletal proteins.

The formation of podosomes and their organization into the belt are critical for efficient bone resorption, as exemplified by the reduced or absent bone resorption by OCs when podosome components are deleted or belt formation is compromised. *Ex vivo* cultures of OCLs from WASP^{-/-} and WIP^{-/-} mice show reduced bone-resorbing activity, as do OCLs when Arp2 or cortactin is depleted *in vitro*. Cortactin's role seems to be central as it is phosphorylated by Src and forms a complex with WIP, N-WASP, and the ARP2/3 complex (Tehrani *et al.* 2007).

SZ-podosome belt formation and stability rely on the microtubule (MT) network. In mature OCs, MTs are organized into two networks, one radial and the other circumferential, each mainly localized at the inner part of the podosome belt. In macrophages podosomes partially co-localize with MTs. Pretreating macrophages with the tubulin polymerization inhibitor nocodazole inhibits podosome formation. In OCLs, the most obvious effect of inhibiting tubulin polymerization is blocking of the transition from clusters and rings to a peripheral belt (Jurdic *et al.* 2006).

The signaling pathways that allow the OC to migrate on bone and form the SZ have been studied in detail. The necessary cell-matrix interaction appears to be principally mediated by integrins, a superfamily of heterodimeric (α and β) transmembrane receptors.

Integrin extracellular domains bind specific peptide sequences in bone matrix proteins, thereby inducing conformational changes and clustering of integrins.

Integrin clustering leads to the formation of adhesion contacts in which integrins are linked to intracellular cytoskeletal complexes, including bundles of actin filaments and signaling molecules (Figure 3.2b). These molecular complexes dictate cytoskeletal rearrangements that regulate podosome assembly and disassembly and allow the OC to spread, move, adhere, or resorb. Interestingly, intracellular signals that are activated by other factors, for example M-CSF, affect the affinity conformation of integrins (Faccio *et al.* 2003).

OCs express high levels of the $\alpha_v\beta_3$ integrin (also referred as the vitronectin receptor) that recognizes an arginine-glycine-aspartic acid (RGD) sequence present on vitronectin, osteopontin, bone sialoprotein, and denatured collagen type I. OCs express integrins including $\alpha_2\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_3$, the third having a central role, as shown by *in vivo* and *in vitro* studies using antibodies against $\alpha_v\beta_3$ or RGD-peptidomimetics that caused reduced resorption. Further evidence for the role of $\alpha_v\beta_3$ in OC function was provided by the targeted deletion of the β_3 subunit in mice that induced late-onset osteopetrosis (McHugh *et al.* 2000). OCs lacking the integrin fail to spread and show defective adhesion and migration, and the formation of the podosome belt is compromised. $\alpha_v\beta_3$ is peripherally located in individual podosomes in mature cells, a localization dependent on the cytoplasmic domain of the integrin.

Integrins signal through changes in intracellular calcium, tyrosine, or serine phosphorylation of signaling molecules and phosphoinositide metabolism, and following $\alpha_v\beta_3$ activation, several pathways are activated. First, activation of $\alpha_v\beta_3$ induces a local increase in cytosolic free Ca^{2+} , which then activates the nonreceptor tyrosine kinase Pyk2, a member of the focal adhesion kinase family. The autophosphorylation of Pyk2 creates a binding site for another tyrosine kinase, c-Src, and activates its kinase activity. One of the targets of c-Src is the adaptor protein c-Cbl that, once phosphorylated, binds and activates a third kinase, PI3K (Horne *et al.* 2005). c-Cbl is also an E3 ubiquitin ligase that polyubiquitylates c-Src and leads to degradation of the Pyk2/c-Src/c-Cbl complex in the proteasome, thereby shutting off the activated signaling pathway. Generation of the c-Src/c-Cbl complex might regulate podosomes assembly and disassembly and affect the shifting of OCs between migration and bone resorption. C-cbl also competes with HDAC6, a tubulin deacetylase, for binding to MTs and thus affects their acetylation. Activated PI3K translocates to the membrane or the actin cytoskeleton, regulating cell attachment and spreading. PI3Ks are a class of enzymes that phosphorylate phosphatidylinositol and its derivatives and modulate their relative levels. PI3K inhibitors disrupt the podosome belt and inhibit

attachment, spreading, and bone-resorbing activity. Deficiency of the p85, α subunit of PI3K, results in increased bone volume in vivo due to decreased OC function (Munugalavadla *et al.* 2008).

Tyrosine phosphorylation of Pyk2 is increased upon ligation of β_3 integrins, but it is only slightly reduced in β_3 -deficient OCs, in contrast with c-Src and c-Cbl (Sanjay *et al.* 2001; Faccio *et al.* 2003). Pyk2^{-/-} mice are osteopetrotic with an increased number of OCs yet in vitro resorption is reduced. Deficiency in Pyk2 disrupts podosome belt formation and increases Rho activation, which in turn affects MTs acetylation via HDAC6 (Gil-Henn *et al.* 2007).

The nonreceptor tyrosine kinase c-Src is one of nine SFKs that share similar structural organization. c-Src is ubiquitously expressed and involved in cell proliferation, growth, adhesion, and migration. However, the dominant phenotype of c-Src-deficient mice is osteopetrosis caused by the failure of OCs to resorb (Soriano *et al.* 1991), identifying c-Src as critical for OC function. OCs also express other SFKs (c-Fyn, c-Lyn, Hck, Fgr), and but with the exception of Lyn, deleting any one of the other SFKs does not generate an obvious bone phenotype. In contrast to the loss of c-Src, deleting Lyn increases RANKL-induced osteoclastogenesis, resulting in osteopenia, but it has little effect on the activity of mature OCs (Kim *et al.* 2009). Although eliminating both c-Src and Hck results in more severe osteopetrosis than in c-Src-deficient mice, it is clear that c-Src performs some specific functions in mature OCs. c-Src is both an adapter protein that links other signaling proteins in complexes and a tyrosine kinase that phosphorylates some components of these signaling complexes. Many of Src's binding partners, including FAK, Pyk2, c-Cbl, paxillin, cortactin, vinculin, talin, and p130^{Cas}, are present in podosomes or involved in integrin signaling, and c-Src therefore plays a central role in regulating dynamic podosome formation and activity. The constitutively active oncogenic Src mutant v-Src induces the formation of podosomes in cell types that do not normally form podosomes, and in OCs, c-Src promotes the initiation of podosome formation, podosome assembly, and the organization of the peripheral podosome belt (Destaing *et al.* 2008). Src-deficient OCs fail to adhere and spread properly or to form a SZ when plated on bone or a podosome belt when plated on glass, and resorb little or no bone in vitro (Horne *et al.* 2005). They are 50%–60% less mobile than WT OCs (Sanjay *et al.* 2001).

Engagement and activation of $\alpha_v\beta_3$ also induce the interaction of c-Src with Syk and the ITAM-bearing proteins DAP12 and FcR γ 1. This complex then activates the Rho GTPase guanine nucleotide exchange factor (GEF) Vav3 (Zou *et al.* 2007). The regulation of cytoskeletal

organization by Rho GTPases is discussed later in this chapter. In vitro, Syk-deficient OCs are almost completely incapable of resorption (Mocsai *et al.* 2004) and their spreading is reduced. Syk also interacts with c-Cbl and, as with c-Src, the interaction results in ubiquitination and degradation of Syk. The global deletion of Syk in mice causes perinatal lethality, but the generation of bone marrow chimeras confirmed the role of Syk in OC function (Zou *et al.* 2007). Interestingly, many of these effector proteins (Syk, DAP12, and FcR γ 1) are also critical for RANKL-induced OC differentiation.

Rho GTPases are key regulators of the actin cytoskeleton and affect numerous cellular processes, including cell polarity, migration, adhesion, vesicle trafficking, and cytokinesis. Mammalian Rho GTPases comprise a family of more than 20 intracellular signaling molecules, including RhoA, Rac1, and Cdc42. Rho GTPases switch between an active GTP-bound state and an inactive GDP-bound state, with cycling between the two states controlled primarily by GEFs. These catalyze the exchange of GDP for GTP in response to diverse extracellular stimuli, and by GTPase-activating proteins (GAPs) that increase the relatively slow intrinsic GTPase activity of Rho proteins (Rossman *et al.* 2005). When bound to GTP, Rho GTPases interact with and activate several effector proteins that affect the actin cytoskeleton dynamics, including WASP, mDIA2, PAK, and the WAVE complex.

Inhibition of Rho GTPases has shown that the activity of these GTPases is required for normal podosome assembly, OC resorption, and mobility in vitro. Inhibition of RhoA downstream of Pyk2 is required for podosome belt formation. Targeted deletion of Rac1, Rac2, or both in the monocyte lineage results in a mild osteopetrotic phenotype (Wang *et al.* 2008). The targeted deletion of Cdc42 in mature OCs results in osteopetrosis, while a mouse model expressing constitutively active Cdc42 exhibits increased bone resorption (Ito *et al.* 2010).

GEF-induced GTPase activation has an important role in promoting OC activity. More than 70 GEFs have been identified in humans, some specific to one GTPase and others not. The three mammalian Vav proteins (Vav 1, 2, and 3) are activated by integrins in multiple hematopoietic cells and activate RhoA, Rac1, and Cdc42 (Rossman *et al.* 2005). In OCs, Vav3 and to a smaller extent Vav1 selectively activate Rac in response to M-CSF, and deleting either or both proteins disturbs the normal regulation of Src phosphorylation. Consequently, Vav3- and Vav1/3-deficient OCs fail to spread, form a podosome belt, and resorb in vitro, and Vav3^{-/-} and Vav1^{-/-}/Vav3^{-/-} mice are osteopetrotic with an increased number of OCs (Faccio *et al.* 2005). Vav3 is phosphorylated in

response to $\alpha_v\beta_3$ ligation, and this response is reduced in the absence of Syk.

Vesicular transport

Bone resorption involves directed vesicular transport toward both the RB, the deeply folded plasma membrane in the area within the SZ facing the bone surface, and the transcytosis domain, part of the plasma membrane on the surface of the OC facing away from the bone.

The RB is the site of different types of directional vesicular transport (Coxon & Taylor 2008). Newly synthesized lysosomal proteins are carried from the trans-Golgi network to form “secretory” lysosomes that express ion channels and transporters on their membranes including the vacuolar H^+ -ATPase (V-ATPase) that acidifies the lumen of the vesicle (Baron *et al.* 1988). The secretory lysosomes also contain a wide variety of soluble hydrolytic enzymes (e.g., CTSK) that operate best at low pH. Thus, this vesicular transport serves both to deliver the ion channels and transporters that directly acidify the resorption compartment to the RB membrane and to deliver catalytic enzymes to the acidified resorption compartment. The resorption compartment can therefore be considered as the equivalent of a giant lysosome. The RB is also the site of intense endocytosis and transcytosis. The resorption process generates large amounts of calcium, phosphate, and degraded matrix proteins that are removed from the resorption compartment via the formation of clathrin-coated vesicles that form at the central area of the RB. Accordingly, this area of the RB is enriched in endocytosis-associated proteins like clathrin, Ap-2, and the GTPase dynamin 2. Interestingly, dynamin 2 is also present in podosomes and associates with several key proteins in integrin signaling, including Src, c-Cbl, and Pyk2 (Bruzzaniti *et al.* 2009). Endocytic vesicles also originate from the BD to form late endosomes that fuse with the RB primarily at the peripheral region of the RB close to the SZ. Transcytotic vesicles containing organic and inorganic resorption products form at the RB, pass through the OC, and fuse and leave the cell at the transcytosis domain. The transcytosis route enables the OC to remove large amounts of degradation products. The transcytosis may also serve to degrade bone components further, since these vesicles are CTSK- and TRAP-positive, and allow calcium intake into the cell.

Several proteins that play important roles in vesicular trafficking in OCs have been identified. The Rab GTPase family ensures the correct delivery of the different vesicles within the cell by controlling vesicle budding, motility, and fusion. Rab7 localizes at the RB, and depletion of Rab7 affects the polarization of the cell and reduces the delivery of secretory lysosomes to the RB. This leads

to reduced resorption in vitro. PLEKHM1, a protein whose deficiency results in rare forms of osteopetrosis in rats and humans, likely interacts with Rab7 for its proper localization on the vesicles (Van Wesenbeeck *et al.* 2007). OCs also express several isoforms of Rab3, including Rab3D, and Rab3D-deficient mice are osteopetrotic due to impaired OC activity. Finally, synaptotagmin VII (Syt VII), a vesicular trafficking protein that links the vesicle and its target, is essential for the formation of the RB as it mediates the fusion of secretory lysosomal vesicles to the bone-apposed plasma membrane (Zhao *et al.* 2008). Accordingly, Syt VII-deficient OCs fail to generate ruffled borders or resorb bone.

The functions of both Rab and Rho GTPases are inhibited by nitrogen-containing bisphosphonates (BPs), which are among the most widely used drugs in the treatment of diseases with excessive bone resorption such as osteoporosis. Nitrogen-containing BPs bind to and inhibit the activity of farnesyl pyrophosphate synthase, a key regulatory enzyme in the mevalonic acid pathway that is critical for the production of lipids (Russell *et al.* 2008). As such, BPs inhibit critical metabolic pathways of the OC, since these lipids are necessary for post-transcriptional modifications (PTMs) of the GTPases that are required for their proper localization. Accordingly, BPs affect the normal transport of vesicles inside the OC, thereby disrupting the SZ and the RB, and inhibiting osteoclast activity.

Acidification of the resorption compartment

The critical first step of resorbing the bone matrix is solubilizing the hydroxyapatite in which the organic components are embedded, thereby allowing the enzymes not only to operate at optimal pH but also to access their organic substrates. The ability of actively resorbing OCs to generate and secrete the large amounts of H^+ ions needed for this step while at the same time maintaining a normal electrochemical balance requires the combined activities of a number of ion pumps and exchangers (Figure 3.3). In addition to the V-ATPases that transport the protons into the resorption compartment, the OC requires (1) carbonic anhydrase (e.g., CAII), which reversibly converts CO_2 to bicarbonate, to generate the protons; (2) Cl^- transporters to transfer Cl^- into the resorption compartment and maintain electroneutrality across the RB membrane; (3) Cl^-/HCO_3^- exchangers in the basolateral membrane to maintain appropriate intracellular pH; (4) sodium–potassium pumps (Na^+ and K^+ -ATPase); (5) K^+/Cl^- co-transporters; and (6) Na^+/Ca^{2+} and Na^+/H^+ exchangers and Ca^{2+} transporters. Mutation of these components results in reduced acidification of the resorption compartment and may lead to osteopetrosis (Supanchart & Kornak 2008).

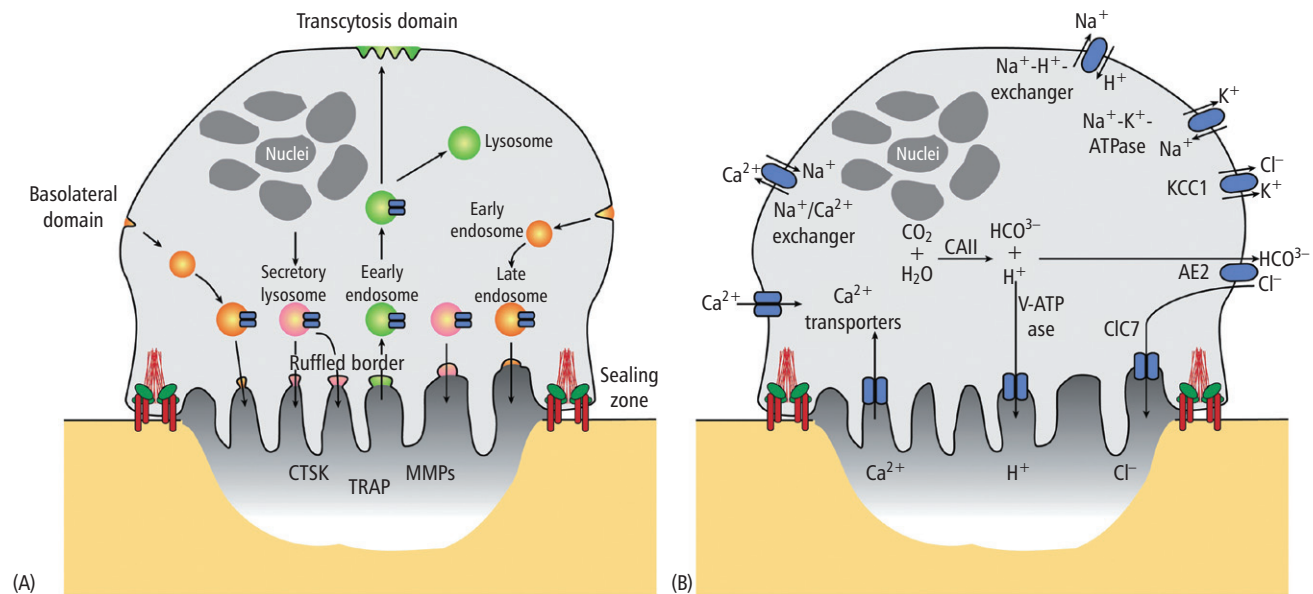


Figure 3.3 A. Osteoclasts are polarized cells with different membrane domains that are the origins or targets of intense vesicular trafficking. B. Osteoclast function relies on various ion channels and transporters that accomplish the acidification of the resorption lacunae, which solubilizes bone matrix minerals (Ca^{2+} and phosphate) and supports the activity of proteases.

The OC RB and late endosomes or lysosomes are highly enriched in V-ATPase complexes, which mediate the extrusion of acid into the resorption compartment. The V-ATPase, like the structurally and functionally homologous mitochondrial F-type ATPase, is composed of two large multi-subunit domains, the soluble ATP-hydrolyzing V_1 domain, and the membrane-associated H^+ -conducting V_0 domain (Forgac 2007). Some subunit isoforms present in the OC RB V-ATPase, notably the α_3 isoform of the large V_0 domain α subunit, are relatively lysosome specific, consistent with the lysosomal nature of the RB membrane. Mutation or deletion of the α_3 subunit disables bone resorption, resulting in osteopetrosis in mice and humans. In addition to its primary role of acidifying the OC resorption compartment, the V_0 domain may also play a role in membrane fusion. OCs that lack the α_3 subunit fail to form an RB, and the absence of another V_0 subunit isoform, α_2 , results in defective fusion of pre-OCs to multinucleated mature OCs (Lee *et al.* 2006). Therefore, the OC V-type H^+ -ATPase complex may be more than just a proton pump.

The RB also contains a H^+/Cl^- antiporter that is composed of α and β subunits, CIC-7 and Ostm1, respectively. Null mutations in either of the subunits disable bone resorption and produce osteopetrosis in both mice and humans (Supanchart & Kornak 2008). Interestingly, the H^+/Cl^- antiporter may contribute more than just maintaining electroneutrality across the RB membrane to enable efficient proton transport. CIC-7-deficient

OCs differentiate normally, but like OCs that lack the V-ATPase α_3 subunit, they fail to form a RB. In addition, elevated Cl^- in the resorption compartment driven by H^+/Cl^- antiporter and the V-ATPase H^+ gradient may contribute directly to bone resorption. A mutation in CIC-7 that uncouples H^+ and Cl^- transport, allowing Cl^- to freely diffuse down the electrochemical gradient established by the V-ATPase but not actively transporting it, still results in osteopetrosis, albeit less severe than that caused by the complete absence of CIC-7 (Weinert *et al.* 2010).

As protons are transported to the resorption compartment, the OC avoids cytoplasmic alkalization by extruding cytoplasmic HCO_3^- via the AE2 Cl^-/HCO_3^- exchanger, a member of the same family as the erythrocyte anion exchanger (AE1). In addition to maintaining cytoplasmic pH in the face of the large secretion of protons, the exchange of cytoplasmic HCO_3^- for extracellular Cl^- provides a source of Cl^- ions that are transported to the resorption compartment. Cytoplasmic Cl^- levels may also be regulated by K^+/Cl^- co-transporters (e.g., KCC1).

Degradation of the bone matrix

Lysosomal enzymes (e.g., TRAP, CTSK, and several metalloproteases) are actively synthesized by the OC endoplasmic reticulum and Golgi network, packaged into secretory lysosomes, and secreted via the RB into the sealed resorption compartment. In the resorption com-

partment, they reach a high extracellular concentration sufficient to degrade the bone matrix. The cysteine protease CTSK is highly expressed in OCs and degrades type I collagen under the acidic pH of the resorption lacunae. CTSK deficiency leads to osteopetrosis in mice (Saftig *et al.* 1998) and pycnodysostosis in humans (Gelb *et al.* 1996). OCs are also rich in acid phosphatase and the tartrate-resistant acid phosphatase (TRACP or TRAP). It is widely used as a marker of OCs. Isoforms of TRAP and collagen peptide fragments and pyridinium cross-links produced by CTSK and other proteases including MMP9 are used as serum and urine markers of bone resorption. In addition to its function as a phosphatase, TRAP also generates reactive oxygen species (ROS), which facilitate the degradation of collagen. However, the contributions of TRAP to bone resorption and the osteopetrosis of TRAP^{-/-} in mice are modest (Hayman *et al.* 1996).

Other roles of osteoclasts

Interaction with bone marrow mesenchymal cells

Bone remodeling occurs throughout life, and the local balance of bone resorption by OCs and bone formation by OBs maintains bone mass in adults. In many pathological conditions such as osteoporosis, rheumatoid arthritis, and periodontitis, this balance is deregulated, and the amount of bone resorbed by OCs exceeds the amount laid down by OBs, resulting in bone loss. It has long been thought that this balance is maintained by the coupling of the activities of these two cell types (Martin & Sims 2005). There is abundant evidence of mutual regulation of osteoclastic and osteoblastic lineage cells in vivo and in vitro and that this interaction is central to OC differentiation. As discussed in this chapter, stromal and osteoblastic cells express M-CSF and RANKL and possibly the ligands for the co-stimulatory receptors OSCAR and TREM2, as well as OPG, the cognate inhibitor of RANKL, and thereby modulate OC differentiation and activity. However, bone remodeling starts with osteoclastic resorption followed by OB formation, suggesting that OCs act on OB precursors and play a role in the promotion of bone formation. This concept of a tight coupling of resorption with formation is implicated in the limitations of most current drugs for bone diseases. In most circumstances, inhibition of bone resorption (by agents like BPs) is followed by reduced bone formation.

In vivo data show that deletion of genes in the OC can affect bone formation. For example, deletion of the calcitonin receptor gene increased bone formation (Dacquin *et al.* 2004). Similarly, deleting Atp6v0d2, a subunit of

the v-ATPase proton pump that is expressed in OCs but not OBs, decreased bone resorption as expected, but it also increased bone formation and OB numbers (Lee *et al.* 2006). Deletion of cathepsin K also increased bone formation (Pennypacker *et al.* 2009).

Some of the mechanisms involved in this coupling have begun to be explained. OCs express the cell-surface molecule ephrinB2 and OB expresses its binding partner EphB4, allowing these molecules to mediate OC–OB interaction (Zhao *et al.* 2006). Signaling through ephrinB2 in OCs inhibits the c-fos-NFATc1 cascade and suppresses OC differentiation, whereas signaling through Eph4 in OBs enhances differentiation. The ephrin–Eph bidirectional signaling thus links OC resorption with OB formation. OCs also secrete factors that promote OB activity. Treatment of OB precursors with conditioned media from OC cultures increases mineralization nodule formation, a marker of OB activity in vitro (Pederson *et al.* 2008). At least three OC-derived coupling factors contained in the conditioned media have been identified: (1) sphingosine phosphate (S1P), the product of sphingosine kinase 1 (SPHK1); (2) BMP6; and (3) Wnt10b. It is also becoming clear that factors present in the bone matrix and released by resorption promote OB formation. TGF- β released during bone resorption coordinates bone formation by inducing migration of bone marrow stromal cells to the bone-resorptive sites (Tang *et al.* 2009). OCs release IGF-I from the bone matrix, a growth factor normally degraded by CTSK (Fuller *et al.* 2008).

Interactions with immune cells

The immune and skeletal systems share a great number of regulatory molecules, including cytokines, receptors, signaling molecules, and transcription factors, as well as a common environment, the bone marrow, where hematopoietic stem cells reside and immune cells form. Because abnormal or excessive bone resorption is frequently associated with a pathological immune response (as happens in rheumatoid arthritis and periodontitis), the modulation of OC function by immune cells through the expression of key osteoclastogenic cytokines has received much attention. Indeed, both T and B lymphocytes secrete RANKL and are important sources of cytokines during inflammatory bone diseases. Other cytokines produced by T lymphocytes and macrophages that affect OCs include IFN γ , IL-4, -6, -10, and -17 (Takayanagi 2007). In contrast with most cytokines identified, IFN γ negatively regulates OC differentiation and RANKL signaling by inducing the degradation of TRAF6. It is likely that T cells present in rheumatic or periodontal inflammatory infiltrates represent a specialized subset of T cells that secrete a specific array of cytokines.

The facts that lymphocytes can influence OCs and that a certain amount of plasticity exists between DCs—antigen-presenting cells (APCs) that activate lymphocytes—and OCs raises the intriguing possibility of a bidirectional interaction that could also affect lymphocytes. Indeed, OCs can function in vitro as APCs and activate T cells (Li *et al.* 2010). Memory T lymphocytes also preferentially home to the bone marrow and proliferate there, and the antigen-presenting ability of osteoclasts could be involved in T lymphocyte homeostasis. Future work will determine the physiologic importance of such a mechanism and a possible role of OCs in immune responses.

Finally, recent studies have indicated that OBs and OCs are involved in the maintenance of the pool of hematopoietic stem cells that reside in a specialized bone marrow microenvironment (the “niche”). Modulating OC activity in the bone marrow affects the mobilization of progenitor cells and their circulation in the blood (Kollet *et al.* 2006). This could potentially affect their availability for different organs and link bone remodeling with the regulation of hematopoiesis.

Conclusion

The last 15 years have seen significant advances in our understanding of the mechanisms by which OCs differentiate and resorb bone, and numerous potential drug targets have been identified to control OC number and activity. Among them, the RANK–RANKL–OPG signaling pathway is central for OC differentiation, and antibodies against RANKL are now in clinical use to treat pathological bone resorption. Previously unrecognized roles of OCs have also emerged, suggesting that OCs participate in a wide variety of biological processes, such as bone formation, immune response, stem cell homeostasis, and even possibly glucose metabolism (Ferron *et al.* 2010). Because of these numerous roles, strategies to reduce pathological bone resorption must be carefully designed. Future drugs not only will have to reduce bone resorption effectively, but also will have to not alter or even positively affect other biological processes, including bone formation.

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4

Clinical correlate: osteopetrosis

Paul C. Edwards and Nasser Said-Al-Naief

Osteopetrosis (OP) is referred to by the eponym *Albers-Schonberg disease*, after the German radiologist Heinrich Albers-Schonberg who is credited with first describing the condition in 1904 (Albers-Schonberg 1904). Osteopetrosis, also referred to by the descriptive term *marble bone disease*, comprises a rare heterogeneous group of inherited diseases of varying severity characterized by defective osteoclast-mediated bone resorption resulting in impairment of normal bone turnover and predisposing patients to increased bone fragility, osteomyelitis, and loss of function of normal medullary bone marrow constituents.

The Nosology Group of the International Skeletal Dysplasia Society (Superti-Fuga & Unger 2007) lists 15 distinct clinical-genetic-phenotypic osteopetrosis-like subtypes under the broad category of genetic bone diseases characterized by “increased bone density without modification of bone shape.” Clinically, OP is commonly classified as having two major types, each with additional subtypes: the autosomal dominant (“benign”) types (corresponding to Albers-Schonberg disease), typically associated with onset in adulthood and mild clinical signs and symptoms, and the severe neonatal or infantile autosomal recessive (“malignant”) types that typically result in death during infancy if untreated. A subtype of the infantile type, referred to as the intermediate form, also presents during childhood with several of the signs of malignant osteopetrosis but with variable morbidity and an intermediate impact on life expectancy (Figure 4.1).

Autosomal recessive OP may also be recognized in the early neonatal period in patients with carbonic anhydrase II deficiency and renal tubular acidosis. This

subtype may be associated with central nervous system abnormalities (e.g., cerebral calcifications), apathy, and hypotonia and muscle weakness secondary to renal tubular acidosis. The osteosclerotic changes and effects on skeletal modeling diminish spontaneously rather than propagating with increasing age (Sly *et al.* 1983; de Verneoul & Kornak 2010).

Numerous mouse models of osteopetrosis have been identified, most resulting from targeted gene mutations in different aspects of osteoclastic development (M-CSF, M-CSF receptor), maturation (RANK, RANKL), or function (TRAP, carbonic anhydrase, chloride channel, cathepsin K). GM-CSF knockout mice are not osteopetrotic, whereas M-CSF knockout mice develop OP, produce less offspring, and have severe cortical electrophysiological abnormalities (McCauley 2001; Helfrich 2003). Human patients with osteopetrosis resulting from M-CSF deficiency have not been identified to date.

The resulting supra-physiologic bone density resulting from impaired osteoclastic activity predisposes the affected individual to increased bone fragility, increased predisposition to fracture, osteomyelitis, nerve compression, growth retardation, and crowding out of normal medullary bone marrow constituents.

Case presentation

A 14-year-old Caucasian male with a history of OP and schizoaffective disorder initially presented for evaluation and treatment of osteomyelitis of the right mandible. A review of his developmental history revealed that he was the product of an unplanned incestuous pregnancy; his biological father was also his grandfather. His mother

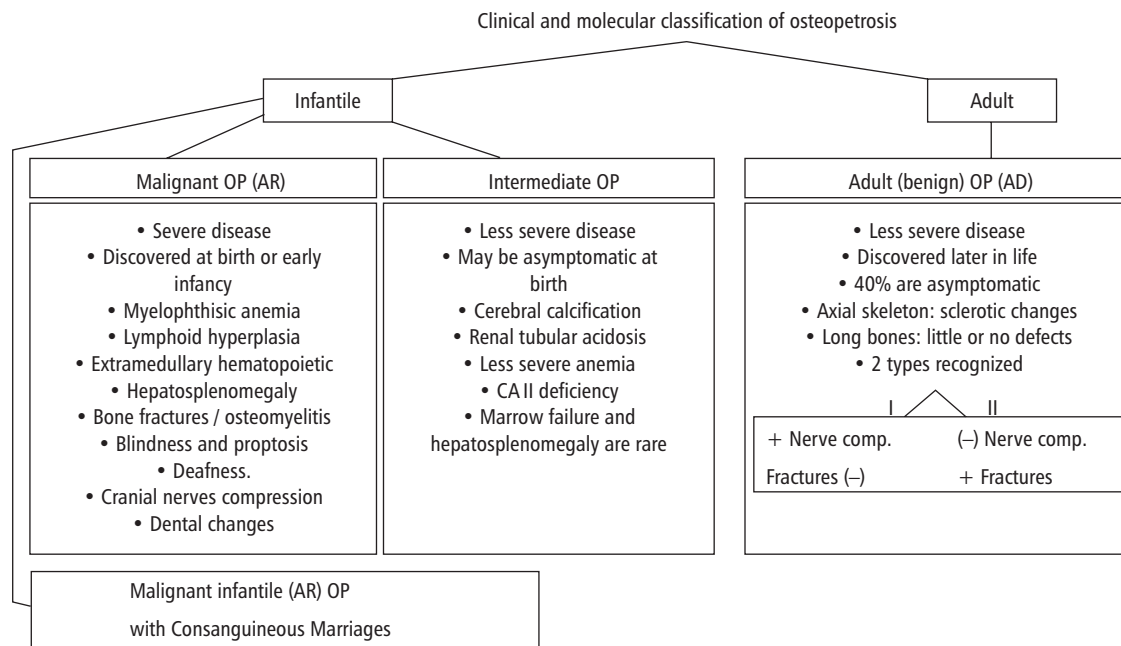


Figure 4.1 Clinical and molecular classification of osteopetrosis.

reported heavy alcohol consumption throughout the pregnancy. He was born prematurely and was generally ill from birth.

He was initially diagnosed with infantile autosomal recessive osteopetrosis, consanguinity related, on day 20 after birth following a confirmatory bone marrow biopsy. He was urgently treated with total body irradiation and busulfan chemotherapy followed by an allogeneic bone marrow transplant.

The patient's medical history was significant for numerous complications related to OP, including anemia, thrombocytopenia, hepatosplenomegaly secondary to extramedullary hematopoiesis, hydrocephalus, cranial nerve palsies, seizure disorder, psychomotor retardation, meningitis, and bone fractures. He was diagnosed with attention deficit hyperactivity disorder and reported multiple psychiatric hospitalizations and two previous suicide attempts while on atomoxetine prior to his presentation for evaluation and treatment of osteomyelitis.

Current medications included oxcarbazepine 600 mg, aripiprazole 5 mg, levothyroxine 50 mcg, and lansoprazole 30 mg daily. He reported allergies to olanzapine, sertraline, and phenylephrine.

His overall health status had remained relatively stable until two years prior to presentation, at which time he started experiencing ear and nose bleeding, headaches, hearing problems, and decreased vision necessitating orbital decompression. He reported a history of delayed tooth eruption and multiple dental restorations. Several months prior to presentation, he had undergone the



Figure 4.2 Radiograph of pelvic bone demonstrating a generalized increase in bone density and loss of medullary space.

extraction of numerous nonrestorable teeth. He reported continued pain and suppuration involving the surgical site on the right mandible.

Clinical examination revealed a pleasant boy of short stature with a broad, trapezoidal head, frontal bossing, and ocular asymmetry. Intraoral examination revealed hypodontia with enamel hypocalcification and a high caries index. Total body radiographic examination revealed generalized increased density of bone with virtually no evidence of marrow space (Figure 4.2). Computed tomography of the head and neck confirmed severe OP of all maxillofacial osseous structures

(Figure 4.3). Panoramic radiography of the maxillofacial structures (Figure 4.4) revealed an extensive area of mottled radiolucency with irregular margins extending from the right incisor region to the medial aspect of the mandibular right second molar. A large central opacity surrounded by a peripheral radiolucency, consistent with an involucrum of bone, was noted within the anterior aspect of the lesion. Diffuse radiopacity was evident throughout the jaws. The final diagnosis was chronic suppurative osteomyelitis in a background of osteopetrotic bone, secondary to surgical tooth extraction.

The patient underwent surgical bone decortication and debridement of the right mandible coupled with

long-term antibiotic therapy (Figure 4.5A–D). His post-surgical course was uneventful. The mandible exhibited good healing with no evidence of non-union or infection. He reported some difficulty with chewing, but reported no other complaints.

Subsequent to the procedure, the patient underwent a second bone marrow transplant and remained relatively stable for the following 10 months, despite suffering from near-total blindness and deafness. He was lost to follow-up due to an out-of-state relocation of the family.

Discussion

This case demonstrates many of the diagnostic approaches, clinical features, and complications associated with OP. As in this case, imaging studies combined with clinical examination are generally necessary to obtain adequate diagnostic information. Features that can be identified radiographically include generalized osteosclerosis, funnel-shaped defects at the metaphyses of the appendicular bones, thickening of the cranial bones, under-pneumatization of the sinuses, and multiple impacted teeth. Evidence of previous fractures or osteomyelitis may also be evident. Imaging studies can be supplemented with genetic testing as needed. Depending upon the underlying genotypic defect, histological examination of affected bone may demonstrate the presence of normal, reduced, or, in the case of OP associated with defects in the acidification process, increased numbers of osteoclasts (Shapiro *et al.* 1988; Helfrich *et al.* 1991; Helfrich 2003).

The clinical differential diagnosis of the osteopetrotic presentation may include Paget's disease, particularly

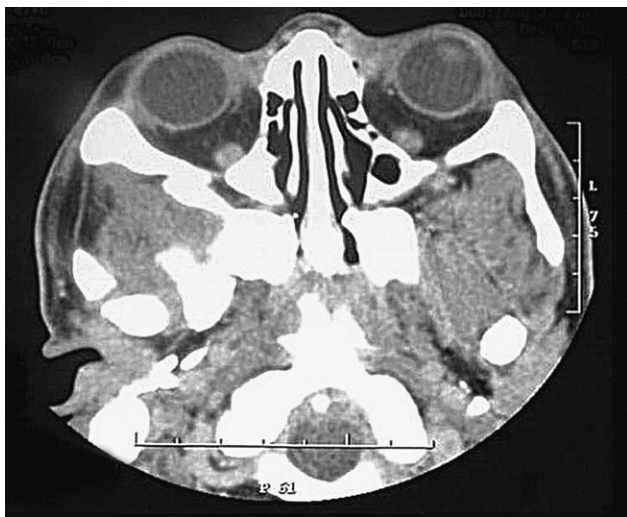


Figure 4.3 Coronal CT scan of the head and neck without contrast demonstrating hyperostosis and marrow obliteration of the maxillofacial bones.



Figure 4.4 Panoramic radiograph demonstrating mottled radiolucency with irregular margins extending from the mandibular central incisor region to the mandibular right second molar. A central opacity consistent with an involucrum of bone is noted within the anterior aspect of the lesion. Diffuse radio-opacity is evident throughout the jaws.

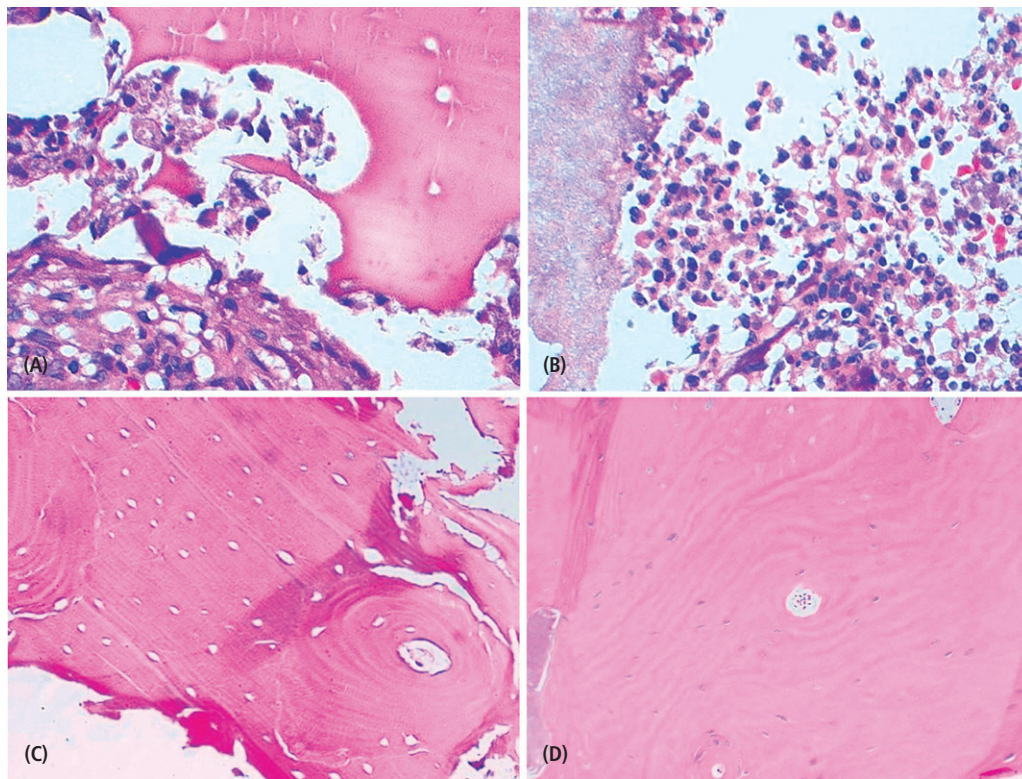


Figure 4.5 A and B: Histomorphological examination of bone removed from the right mandible during surgical debridement reveals necrotic bone (sequestra) juxtaposed to a dense mixed chronic and acute inflammatory cell infiltrate, diagnostic of osteomyelitis (hematoxylin and eosin stain; original magnification $\times 40$). Photomicrographs of bone obtained from the margin of the debrided bone demonstrating replacement of medullary bone by extremely dense bone at (C) low magnification and (D) intermediate magnification (hematoxylin and eosin stain; original magnifications $\times 10$ and $\times 20$, respectively).

with regard to adult-onset OP; myeloid and myeloproliferative disorders such as sickle cell disease, myelofibrosis, and leukemia; metabolic conditions such as hyperparathyroidism and pseudohypoparathyroidism; mastocytosis; renal osteodystrophy; and heavy metal (e.g., lead or beryllium) and fluoride toxicity.

The overall incidence of OP is difficult to estimate, but it is believed to range from 1 in every 250,000 births for the severe infantile autosomal recessive types (Loria-Cortes *et al.* 1977) to 1 in every 20,000 for the milder autosomal dominantly inherited adult-onset types (Bollerslev & Andersen 1988).

Clinical features noted in the craniofacial structures include increased thickness of the cranial vault, frontal bossing, macrocephaly, micrognathia, loss of mandibular angle, craniosynostosis, and loss of vision and hearing resulting from impingement of the cranial nerve foramina. Characteristic dental features include impaired and/or delayed tooth eruption, occasionally resulting in the clinical appearance of “two rows of teeth,” and abnormal dentinogenesis. Osteomyelitis of both the mandible and maxilla following surgical intervention, such as tooth

extraction, is a well-described complication of OP (Bakeman *et al.* 1998; Barbaglio *et al.* 1998; Barry *et al.* 2007; Satomura *et al.* 2007). Common extracranial features include generalized failure to thrive; seizure disorders; hypocalcemia; increased susceptibility to infection, especially of viral origin; and long-bone fractures.

Treatment approaches vary widely due to the underlying heterogeneity of subtypes and variations in the extent of phenotypic expression (Stark & Savarirayan 2009). In many cases, the milder adult-onset OP subtypes may require no specific treatment other than the management of any complications that may develop (e.g., osteomyelitis). Management of the intermediate OP subtypes will also vary, depending on the affected individual's degree and spectrum of involvement. The infantile subtypes typically require aggressive intervention to reduce morbidity and mortality. This generally involves management of hematologic disturbances, which may include the administration of erythropoietin to treat anemia, and the administration of gamma interferon, which may afford improvements in immune function and, to a lesser extent, an increase in bone-resorptive

activity. Interventions to stimulate bone resorption, including the delivery of large doses of glucocorticosteroids and calcitriol, are occasionally employed, although the benefits may only be short term. The mainstay of therapy in the most severe infantile subtypes, those with associated bone marrow failure, involves hematopoietic stem cell transplantation within the first three months of life. Genetic counseling should be considered for the parents of individuals with infantile and intermediate OP subtypes.

Future approaches to therapy (Askmyr *et al.* 2008) may include the introduction of alternative methods for stimulating osteoclastic function (e.g., continuous infusion of parathyroid hormone), the administration of recombinant M-CSF and recombinant RANKL protein, and mesenchymal stem cell transplantation, especially in subtypes characterized by impaired osteoclastic maturation.

The long-term prognosis for patients with OP also differs depending on the underlying phenotypic-genotypic-clinical presentation. Adult-onset OP subtypes are generally associated with an excellent prognosis. While the infantile subtypes of OP have, on average, a good prognosis (a 75% five-year survival rate following hematopoietic stem cell transplantation from HLA-identical siblings), significant morbidity is seen in the majority of affected individuals. Without treatment, early death associated with bone marrow suppression is typical, leading to anemia, increased susceptibility to infection, and bleeding diatheses.

Conclusion

Juvenile-onset osteopetrosis, even when aggressively managed by current approaches such as bone marrow transplantation (BMT), is associated with significant morbidity.

Acknowledgments

We would like to thank Drs. Patrick Louis and Jon Holmes for sharing the radiographic images with us.

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Clinical correlate: *CLCN7*-associated autosomal recessive osteopetrosis

Piranit Nik Kantaputra

Bone is a dynamic tissue in which osteoblasts synthesize bone matrix while osteoclasts direct bone resorption. Bone homeostasis is strictly based on the balance between bone formation and bone resorption. If this balance is disturbed, it can lead to reduced bone quantity and quality (osteoporosis) or abnormal accumulation of bone (hyperostosis, osteosclerosis, and osteopetrosis). Human osteopetrosis (or marble bone disease) is the term that refers to a heterogeneous group of rare genetic bone disorders, characterized by increased bone density that can be seen on radiographs. The increased bone mass is the result of defective osteoclast function or number. Osteoclasts, bone-resorbing cells, are multinucleated cells derived from precursors of the monocyte-macrophage lineages (Wang & McCauley 2011). In osteopetrosis, a decrease or complete failure of bone resorption resulting from abnormal osteoclasts leads to generalized osteosclerosis (dense bone), reduction of bone marrow spaces, and increased brittleness of bones. The manifestations range widely in severity. However, the signs and symptoms are mainly the result of an abnormal osteoclast number or function.

Human osteopetroses include infantile malignant autosomal recessive osteopetrosis (ARO; OMIM 259700), intermediate autosomal recessive osteopetrosis (IARO), and benign autosomal dominant type I (ADO I or OPTA1; OMIM 607634) and type II (ADOII or OPTA2; Albers-Schoenberg disease; MIM 166600) (Pangrazio *et al.* 2010). All forms of osteopetrosis are characterized by defective bone resorption mainly due to abnormal osteoclast function. Infantile malignant ARO is the most severe and lethal form of osteopetrosis, and it occurs in osteoclast-poor and osteoclast-rich forms. Osteoclast-

rich ARO has been reported to be associated with mutations in the *CLCN7*, *TCIRG1* (ATP6V0A3), and *OSTM1* genes. Mutations in *TCIRG1* are responsible for more than 50% of ARO patients (Frattini *et al.* 2000; Kornak *et al.* 2000; Sobacchi *et al.* 2001), *CLCN7* mutations are responsible for approximately 13% of ARO patients (Kornak *et al.* 2001; Frattini *et al.* 2003), and *OSTM1* mutations are responsible for approximately 2% of ARO patients (Pangrazio *et al.* 2006). The clinical phenotypes caused by these mutations are very similar. Patients with ARO usually have CNS involvement, but patients with mutations in *CLCN7* and *OSTM1* have been reported to have more severe CNS symptoms (Kornak *et al.* 2001; Frattini *et al.* 2003; Pangrazio *et al.* 2006).

Oral manifestations in ARO patients include malformed teeth, absence of tooth roots, impacted or unerupted teeth, malpositioned teeth, and jaw osteosclerosis (Dick & Simpson 1972). One of the most common complications found in ARO patients is mandibular osteomyelitis.

In this chapter we present the case of a 25-year-old Thai patient who is affected with ARO that appears to be the result of a *CLCN7* mutation. A complete description of this patient has been published elsewhere (Kantaputra *et al.* submitted).

Case presentation

A 25-year-old Thai man presented to the Oral and Maxillofacial Surgery Clinic, Faculty of Dentistry, Chiang Mai University, with mandibular osteomyelitis. He was the first child of consanguineous Thai parents. His younger sister was not affected. He had been treated in

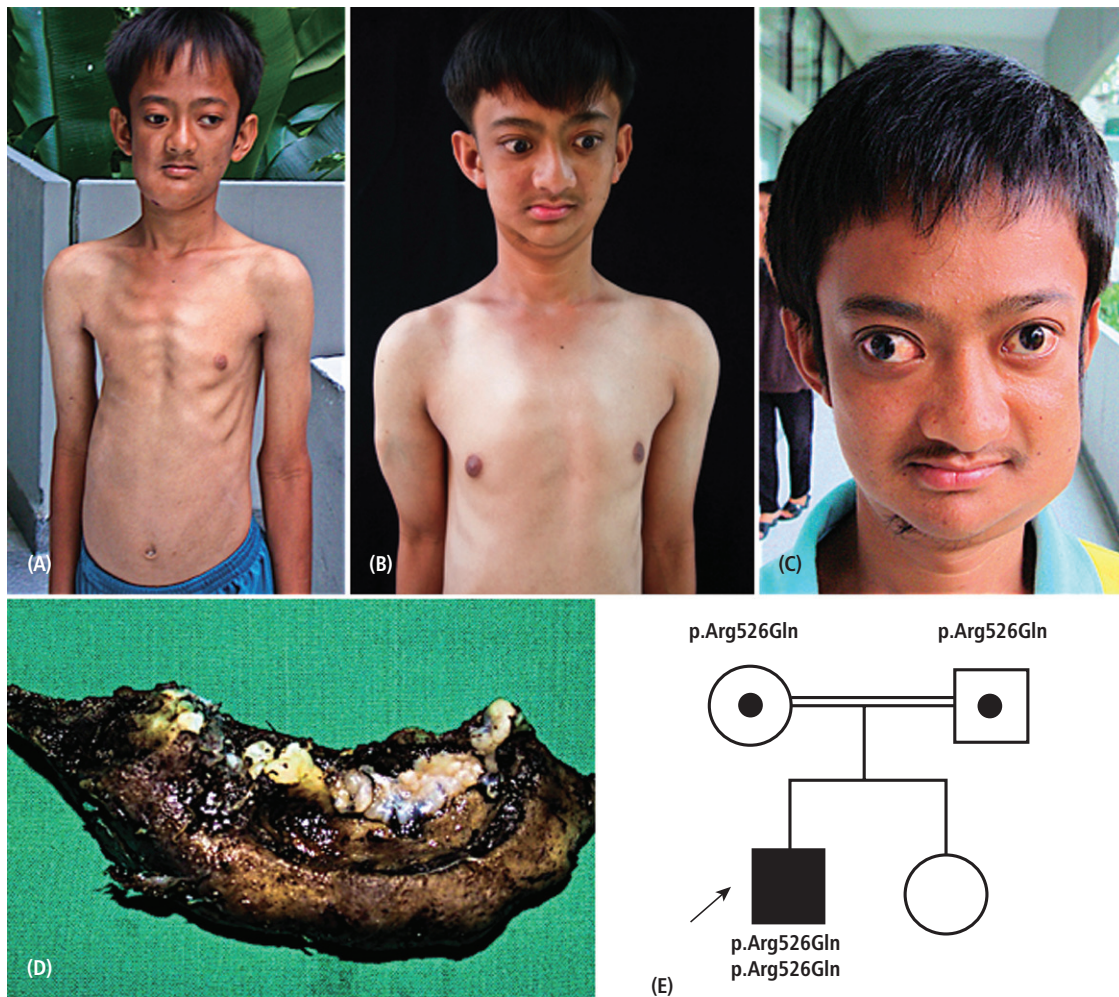


Figure 5.1 A and B. Patient at age 15 and 18 years, respectively. Note frontal bossing, protuberant abdomen caused by hepatosplenomegaly, exophthalmos, and right-forearm deformity. C. Patient at age 23 years. Exophthalmos is more severe. Esotropia and swollen left cheek are evident. D. Patient underwent a partial mandibulectomy. Note the unerupted and malformed teeth. E. Family pedigree.

the Oral and Maxillofacial Surgery Clinic since he was 15 years old (Figure 5.1A–C). Hepatosplenomegaly secondary to compensatory extramedullary hematopoiesis was first detected when he was three months old. Clinical manifestations consisted of proportionate short stature, frontal bossing, esotropia, exophthalmos, and protruded abdomen (Figures 5.1A–C). His intelligence appeared normal as did his hearing. His visual impairment began at six months of age but did not appear to be progressive. As a result of a reduced bone marrow space, he had been anemic since birth. From the age of 15–25 years, his hemoglobin levels ranged from 9.7 to 13.4 gm/dl. His hematocrit level ranged from 30% to 41.5%. White blood cell and platelet counts were unremarkable. He suffered from fractures of the humeri and femur due to the severe brittleness of his bones.

Radiographic examination revealed generalized osteosclerosis of the cranial vault, cranial base, mandible, maxilla, vertebrae, ribs, pelvis, and limbs (Figures 5.2, 5.3, 5.4, 5.5, and 5.6). Bilateral healed humeral shaft fracture deformities were evident (Figure 5.6E). Malformation of the acetabula and femoral heads was noted (Figure 5.6B and 5.6D). Obliteration of the bone marrow was clearly evident (Figures 5.5 and 5.6). The panoramic radiograph showed a number of unerupted, malformed, and abnormally calcified teeth (Figure 5.4A and 5.4B). Absent and rudimentary molar roots were evident, and areas of osteosclerosis were present in the mandible. Calcification of stylohyoid ligaments was observed (Figure 5.4A). Computerized tomography performed at age 24 years revealed a fracture of the mandible, a partial mandibulectomy (Figures 5.2A–D), elongated styloid

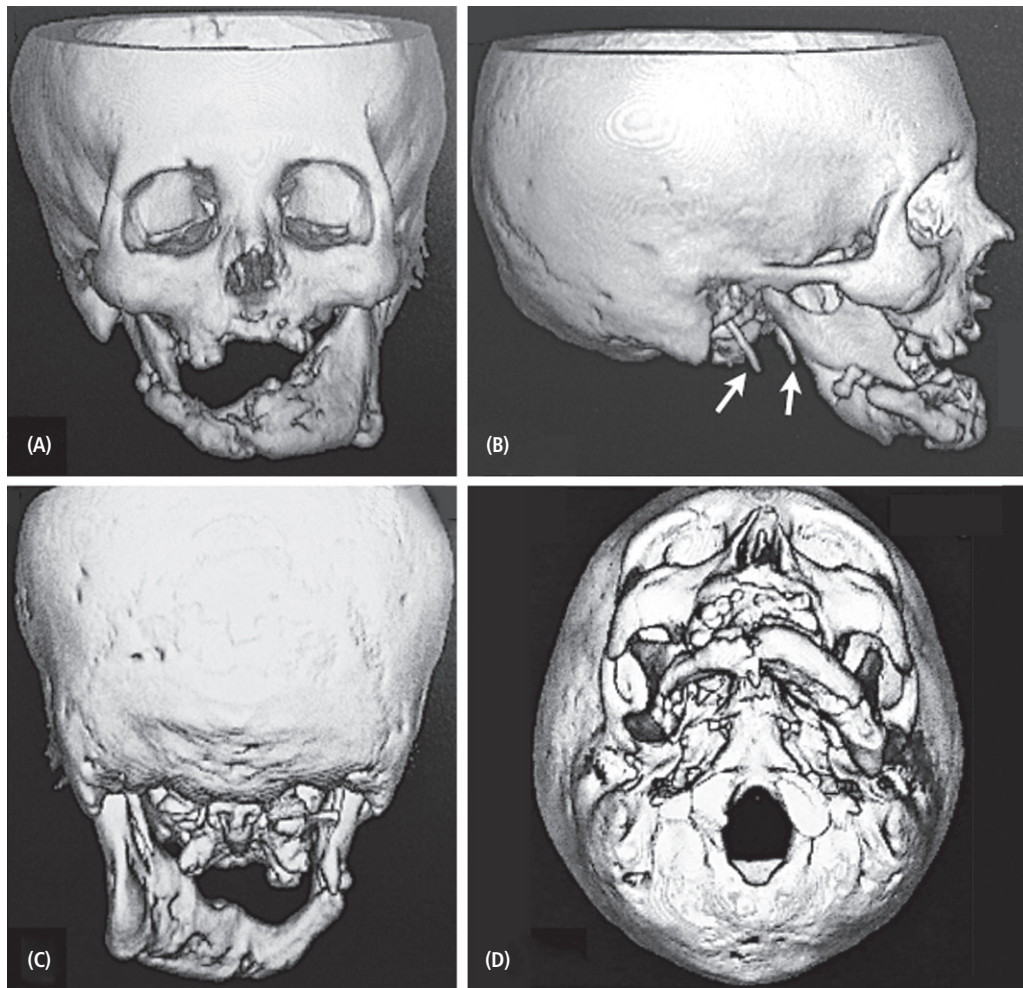


Figure 5.2 Three-dimensional computerized tomography (3D-CT) of the skull taken at age 24 years. Note the fracture of the mandible that was subsequently removed on the right side and the elongated styloid processes (arrows).

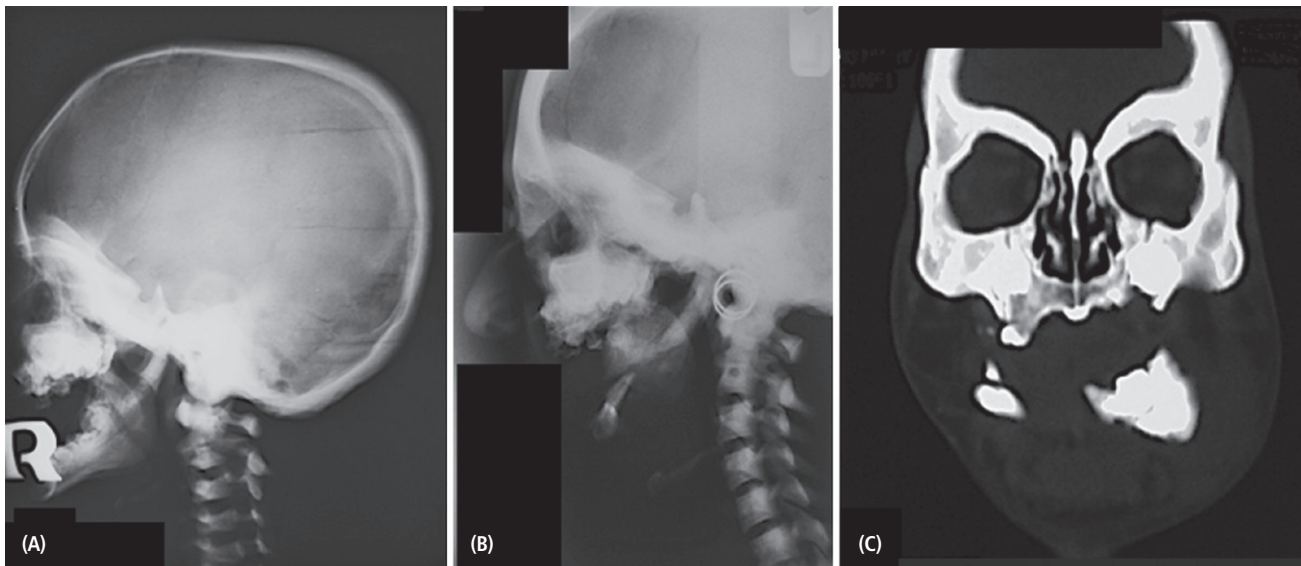


Figure 5.3 Lateral cephalograms taken at age 15 (A) and 25 (B) years, respectively. The cranial base and atlas bone are dense. (C) The cranial vault and posterior clinoid process are much thicker at age 25 years. CT shows obliteration of maxillary sinuses and the mandible after partial mandibulectomy.

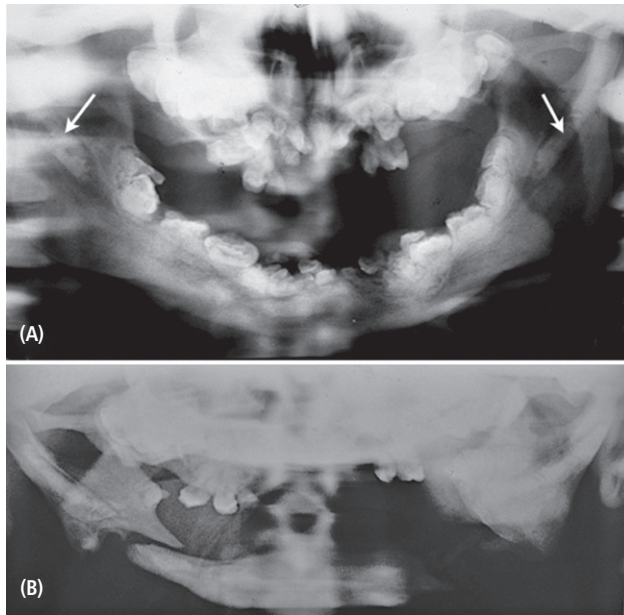


Figure 5.4 Panoramic radiographs of patient taken at age 15 (A) and 25 (B) years. Note the abnormal tooth shapes and severe root malformation. Molar roots are rudimentary, and there is crowding of unerupted teeth. The arrows indicate calcified stylohyoid ligaments. Note also the narrow body of the mandible and the fracture of mandible on the right side (B).

processes (Figure 5.2B), and obliteration of the maxillary sinuses (Figure 5.3C).

Between the age of 15 and 25 years, the patient suffered five episodes of mandibular osteomyelitis. Sequestrotomy and curettage were performed initially but were not successful in treating the right side of the mandible. At age 15 years, a partial mandibulectomy of the right side of the mandible was performed under general anesthesia (Figure 5.1D). Choanal stenosis had made nasotracheal intubation impossible. Even though the patient was aware that he had hepatosplenomegaly as a result of compensatory extramedullary hematopoiesis, he drank alcohol every day and became an alcoholic. At age 24 years, he suffered from alcoholic hepatitis and hypertension but refused to receive any treatment.

Mutational analysis of *CLCN7*

Mutational analysis of *TCIRG1* was first performed as described in this chapter, but the pathogenic mutation was not detected (Kornak *et al.* 2000). Bidirectional sequencing of the coding regions and the flanking introns of the *CLCN7* gene of the proband and his parents was subsequently performed (Kornak *et al.* 2001). A homozygous mutation of G>A at nucleotide position 1577 (c.1577G>A) within exon 17 was identified. This mutation is predicted to change a polar, negatively charged amino acid arginine (Arg;R;CGG) to an uncharged polar glutamine (CAG;Gln;Q) at amino acid position 526 (p.Arg526Gln). The patient's father and

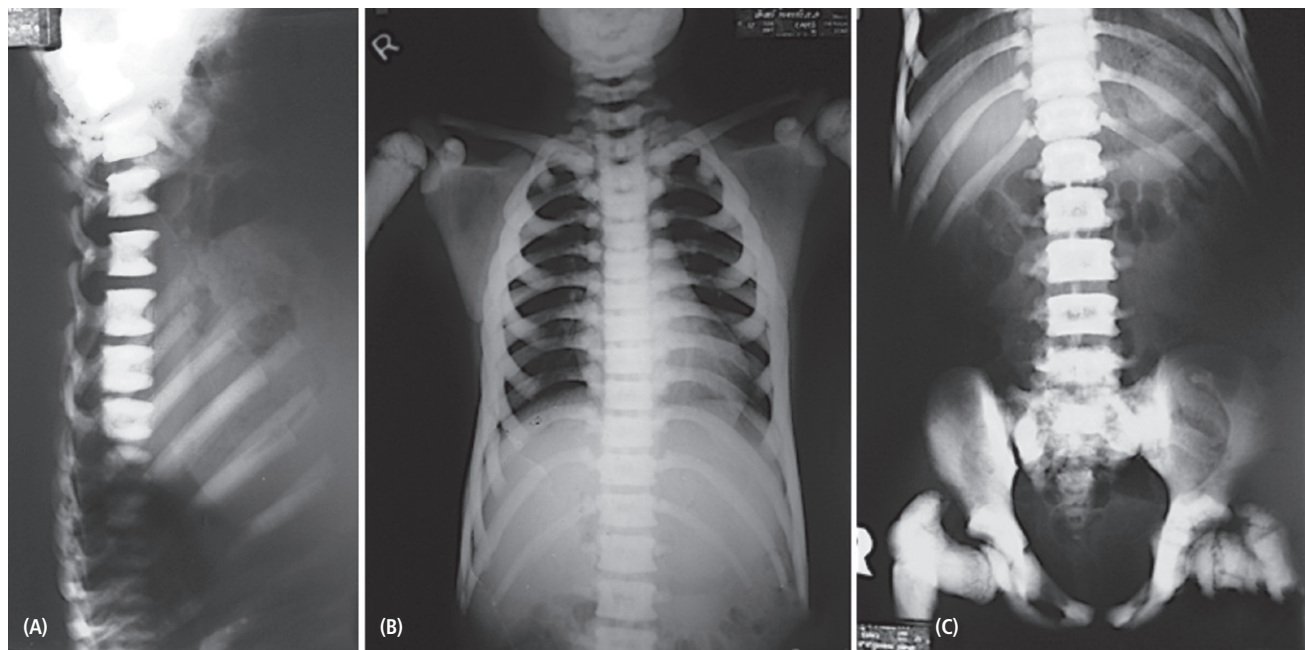


Figure 5.5 A–C. Generalized sclerosis of bones. The upper and lower end plates of the vertebral bodies are dense. Note the malformed acetabula and irregular head of femora (C). Greater trochanters of the femoral heads are prominent. The iliac crests are less sclerotic.

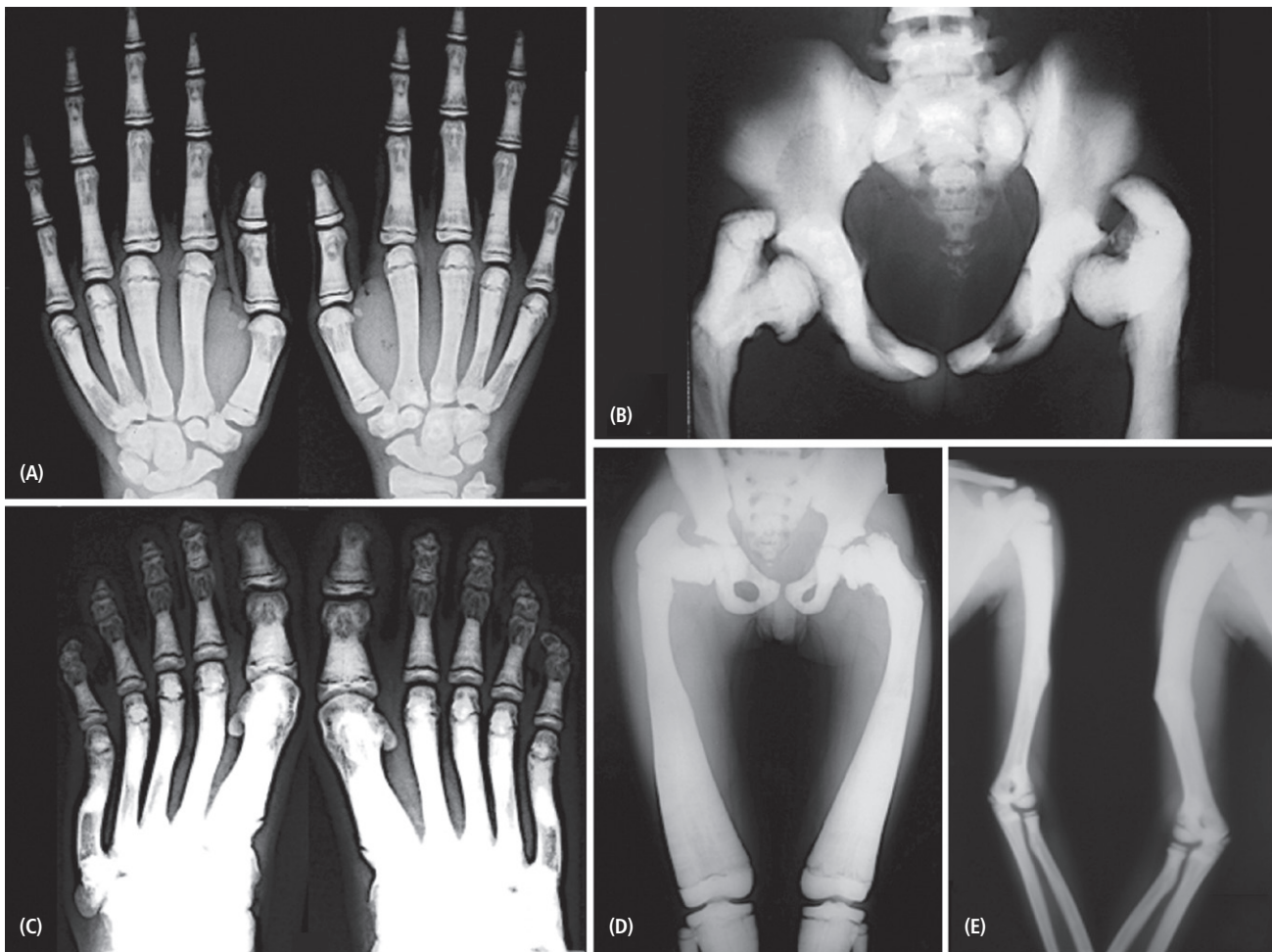


Figure 5.6 A–E. Generalized osteoclerosis. Pelvic radiographs (B and D) demonstrate severely malformed pelvis and femoral heads. Acetabula are abnormally shallow. Superior pubic ramus, inferior pubic ramus, and ischial tuberosity are severely hypoplastic. The iliac crests are less sclerotic. The heads of the femora are sclerotic with irregular foci of sclerosis. Greater trochanters are abnormally prominent. There is metaphyseal clubbing of the femora. Fracture deformities of the humeri are evident on the humeri (E).

mother were found to be heterozygous for the same mutation. This DNA variant was not detected in 100 normal Thai controls.

Discussion

The clinical and radiographic findings of this patient point to a diagnosis of ARO. Infantile malignant ARO is the most severe and lethal form of osteopetrosis, and has been reported to be associated with mutations in *CLCN7*, *TCIRG1*, and *OSTM1*. We initially sequenced *TCIRG1* as it is the most common gene associated with ARO but we did not find a pathogenic mutation in *TCIRG1*. Subsequently, we found a homozygous missense mutation (p.Arg526Gln) in *CLC-7*. Mutations in *CLCN7* cause a spectrum of osteopetrotic phenotypes including ARO, IAO, and ADOIL. Heterozygous mutations in *CLCN7* are associated with variable expressions of the phenotypes

even within the same family, ranging from a nearly asymptomatic form to a very severe form (Frattini *et al.* 2003; Steward 2003; Campos-Xavier *et al.* 2005; Pangrazio *et al.* 2006; Phadke *et al.* 2010).

CLC-7 (MIM 602727) is a member of the mammalian CLC family. The CLC gene family was originally identified by the expression cloning of a voltage-gated Cl⁻ channel CLC-0 from the electric organ of the marine ray *Torpedo marmorata* (Jentsch *et al.* 1990). CLC genes are expressed in species from bacteria to humans and encode Cl⁻ channels or Cl⁻/H⁺ exchangers. Clcn7 is expressed in various cell types in the vesicles of the endocytotic-lysosomal pathway. In osteoclasts, the CLC-7 protein is localized in late endosomal and lysosomal compartments adjacent to the ruffled border, a specialized membrane created by the exocytotic insertion of lysosomal membranes. At the ruffled border of the osteoclasts, the CLC-7 protein colocalizes with its β -subunit Ostm1 and

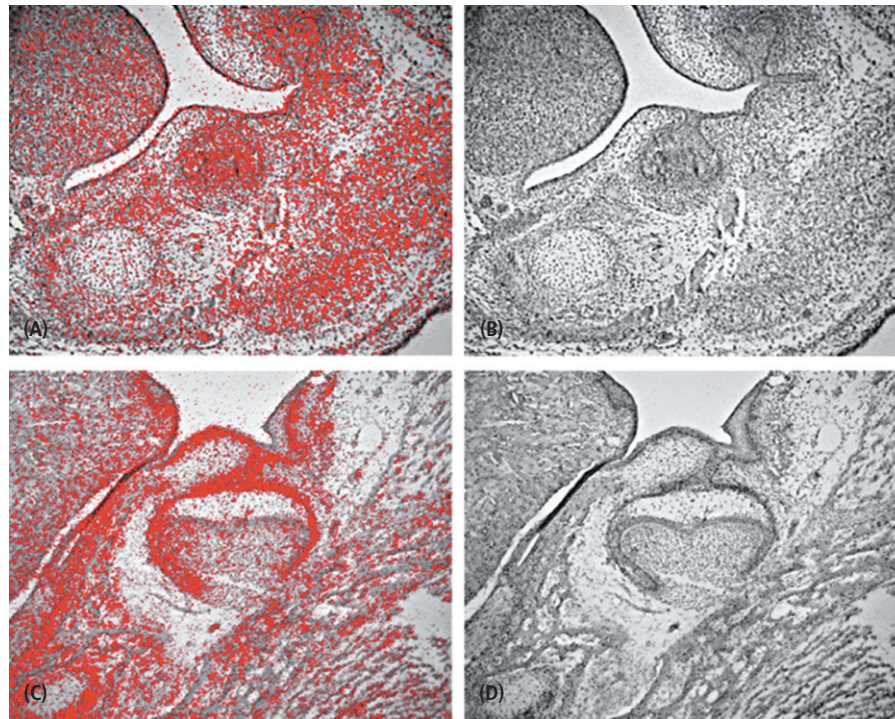


Figure 5.7 *Clcn7* expression in murine tooth development. Radioactive in situ hybridization of *Clcn7* expression on frontal head sections at the positions of the lower first molars. E14.5 (A and B) and NB (C and D). B and D are images of bright field of A and C, respectively. *Clcn7* is weakly expressed in tooth germs and alveolar bone at E14.5. Strong *Clcn7* expression is observed in dental lamina and outer enamel epithelium at birth. (Figures are courtesy of Dr. Atsushi Ohazama and Professor Paul Sharpe.)

the $\alpha 3$ subunit of the V-type H^+ -ATPase (Lange *et al.* 2006). CLC-7 and *Ostm1* have specific interactions with each other. It has been demonstrated that *Ostm1* needs CLC-7 to reach lysosomes, but CLC-7 does not need *Ostm1* to reach that compartment.

CLC-7, like other CLC proteins, assembles to dimers, with each monomer containing an ion translocation pathway. Some mammalian isoforms comprise essential β -subunits (barrtin and *Ostm1*). CLC-7 is the only mammalian CLC protein that is predominantly expressed in the lysosomal membrane (Jentsch 2008). Its function is to extrude chloride anions (Cl^-) in exchange for HCO_3^- in order to neutralize the electric current generated by the H^+ -ATPase. This exchange takes place through an anion exchanger located in the basolateral membrane, leading to continued availability of Cl^- for acidification of the resorption lacuna (Jansen *et al.* 2009; Wang & McCauley 2011).

The patient exhibited dental and jaw anomalies including malformed and malpositioned teeth and impacted or unerupted teeth. Osteomyelitis of the jaws and absence of molar roots are frequent findings in ARO patients (Krithika *et al.* 2009). *Clcn7* is highly expressed in alveolar bone and the outer dental epithelium (Figures 5.7A and 5.7B). The outer dental epithelium plays an

important role in root development. This might explain the absent or rudimentary tooth roots and the failure of tooth eruption in ARO patients. Since *Clcn7* is strongly expressed in the alveolar bone, osteomyelitis of the jaws is postulated to be the consequence of *CLCN7* mutations (Kantaputra *et al.* in press). The calcification of stylohyoid ligaments found in the patient demonstrated the similarities that these ligaments share with bones.

In vitro osteoclastogenesis has demonstrated that p. Arg526Gln mutation of CLC-7 did not have any effect on multinuclear cell formation. The morphology of ARO osteoclasts was irregular and distinctively larger than that of the heterozygous carrier or the controls (Figures 5.8A–C; Kantaputra *et al.* submitted). The hypertrophic appearance might be the result of the alteration of intracellular ion physiology under non-apoptotic conditions (Okada *et al.* 2006). However, a recent study has proposed that if mutant osteoclasts do not resorb bone, they will not become fully differentiated (Neutzsky-Wulff *et al.* 2010). The ARO osteoclasts from the patient became very large with large numbers of nuclei, but F-actin rings were absent. This might indicate that CLC-7-deficient osteoclasts have an increased tendency to undergo cell fusion. How this might be related to its function as an ion exchanger is currently not under-

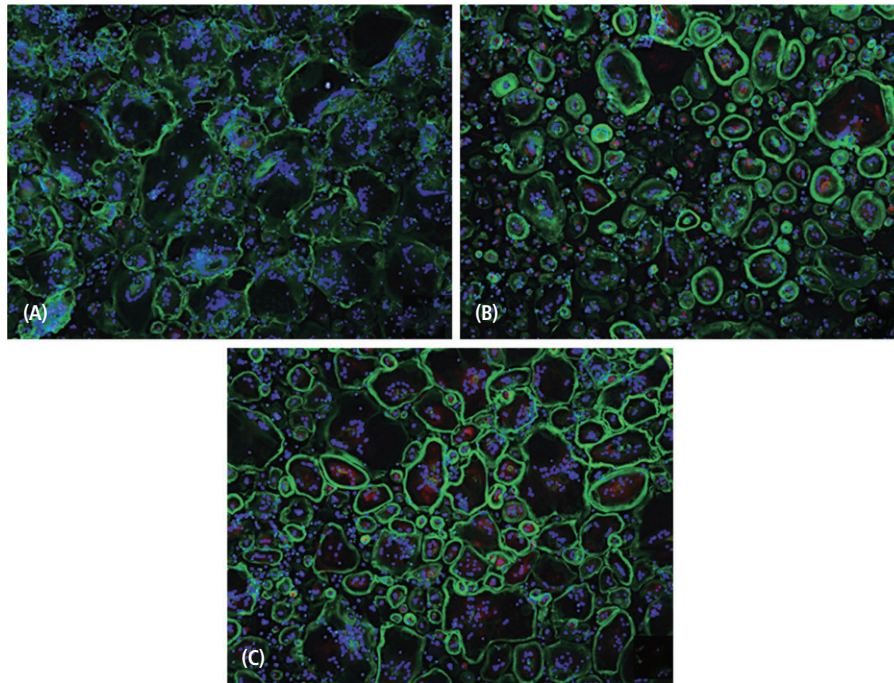


Figure 5.8 The in vitro differentiation of (A) homozygous p.Arg526Gln CLCN7, (B) heterozygous carrier, and (C) sex-matched control osteoclasts. Osteoclasts are stained with DAPI (blue), F-actin conjugated phalloidine (green), and TRAcP (red). (Figures are courtesy of Dr. Chayarop Supanchart.)

stood. Osteoclast differentiation and function are also important for the regulation of osteoblast activity. Thus, osteoclast dysfunction may also affect bone formation during bone remodeling (Karsdal *et al.* 2008; Neutzsky-Wulff *et al.* 2010). In addition to abnormal bone resorption, abnormal bone formation might partly be responsible for the overall sclerotic bone phenotype seen in *CLCN7*-associated osteopetrosis patients.

Conclusion

The patient was a 25-year-old Thai man with ARO who had a homozygous missense mutation in *CLCN7*. His clinical and radiographic features are characteristic of ARO. This patient appears to be the longest lived ARO patient ever reported. It is interesting to note that this patient has never had a bone marrow transplantation and drank alcohol every day for eight years yet still has survived beyond the maximal life expectancy by many years. The mutant osteoclasts generated from his peripheral blood mononuclear cells were very large and were shaped irregularly with poorly demarcated circular F-actin. Lack of resorptive function was demonstrated. Besides retinal degeneration, it appears that the phenotype of patients with osteopetrosis is secondary to the abnormal osteoclasts.

Acknowledgments

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6

Osteoblasts of craniofacial bone

Renny T. Franceschi, Chunxi Ge, and Christopher G. Wilson

As the cells responsible for secretion and mineralization of the extracellular matrix of bone, osteoblasts are essential for maintaining the structural integrity of the skeleton. An understanding of their origins, development, and function is a prerequisite for explaining genetic and acquired craniofacial disorders as well as for repairing or regenerating cranial bones. This chapter will discuss the origin of craniofacial osteoblasts from the cranial neural crest, summarize the transcriptional mechanisms that control osteoblast differentiation, provide an overview of the main biological functions of osteoblasts, and describe how these cells are controlled by several humoral and local signals. Lastly, the use of osteoblasts and osteoprogenitors for craniofacial regeneration will be reviewed.

Embryological origin of craniofacial osteoblasts from neural crest and specification of cellular fate

Cranial neural crest cells (NCCs) originate in the anterior-dorsal aspect of the developing neural tube and contribute to most of the cartilage and bone of the cranial region (Figure 6.1). The most rostral cranial NCCs arise from the diencephalic and mesencephalic neural tube to form the frontonasal skeleton and the membranous bones of the skull. The more posterior cranial NCCs coming from rhombomeres of the posterior mesencephalon and hindbrain occupy the pharyngeal arches where they form the mandible, maxilla, middle ear bones, hyoid, and thyroid cartilages. It is thought that the various craniofacial morphologies across species and even within a species (such as individual facial characteristics) are largely determined by

the controlled deposition of bone by neural crest–derived osteoblasts. The positional identity of NCCs is defined by a combination of an intrinsic anteroposterior pattern of homeobox (Hox) gene expression and gradients of diffusible molecules that control levels of other homeodomain (HD) transcription factors. For a review, see Minoux and Rijli (2010).

Two HD genes, *Emx1/2*, define anteroposterior patterning of head mesenchyme and *Otx1/2*, while patterning of the pharyngeal arches is controlled by members of the Hox gene family (Hox a2/b2, Hoxa3/b3/d3, and Hoxd3/d4). Hox expression is originally established in the rhombomeres (from which the NCCs originate) and is then modified as each NCC population migrates to a pharyngeal arch. Within the pharyngeal arches, dorsal-ventral as well as anteroposterior axes are further defined by diffusible factors such as endothelin 1 (EDN1), sonic hedgehog (SHH), fibroblast growth factors (FGF), and bone morphogenetic proteins (BMPs) that are released from specific signaling centers within the developing craniofacial region. The positional information provided by these gradients induces a distinct pattern of homeodomain transcription factor expression to give each craniofacial region a specific signature. For example, EDN1 is secreted by the surface ectoderm of pharyngeal arches to form a ventral-dorsal gradient that differentially activates a third class of HD genes, the *Dlx* family (*Dlx1–6*), to control dorsal-ventral patterning of cells within each arch. The HD protein expression signature of each region is thought to specify the particular pattern of bone formation for that region, for example, specification of maxillary or mandibular structures in the first pharyngeal arch. For more on this subject, see Chapter 1 in this volume.

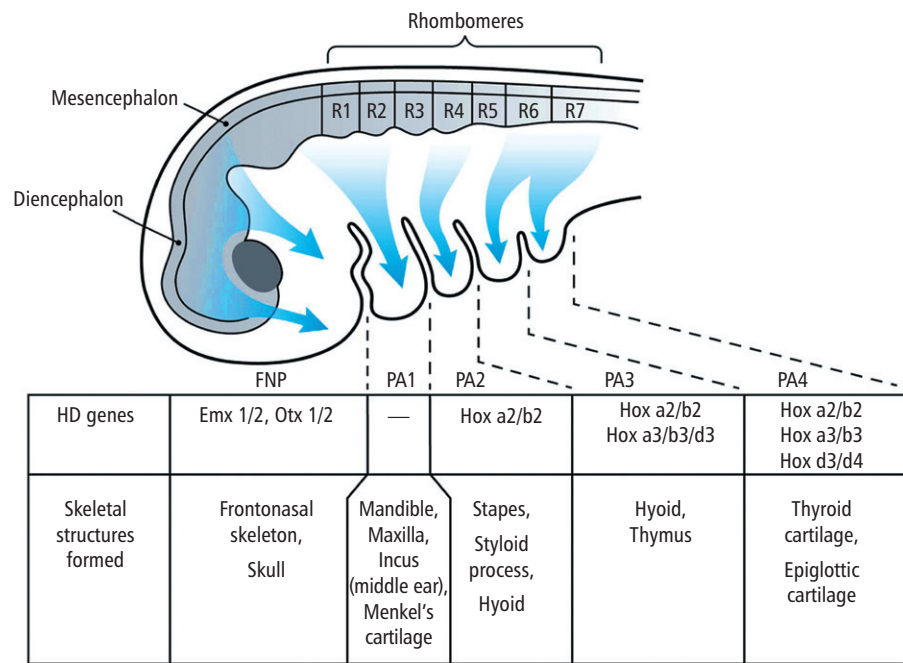


Figure 6.1 Embryological origin of craniofacial osteoblasts from the neural crest. Proposed migratory routes of neural crest cells are shown from diencephalic and mesencephalic regions and anterior rhombomeres. Frontonasal process (FNP) and pharyngeal arch (PA1–PA4) destinations of each neural crest population are indicated as well as the specific homeodomain genes involved in the specification of each region. (Adapted from Minoux & Rijli, 2010.)

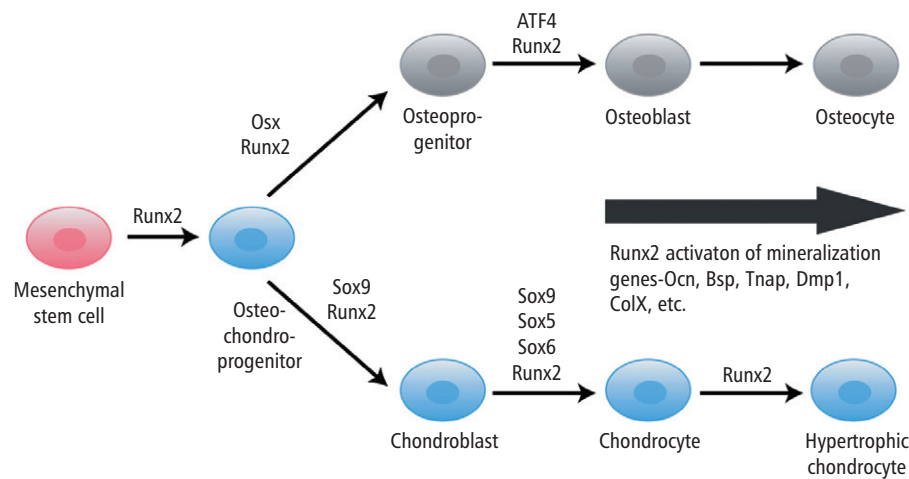


Figure 6.2 Major transcription factors controlling skeletal lineages. The differentiation of mesenchymal stem cells to osteoblast–osteocyte and chondroblast–hypertrophic chondrocyte lineages is shown with sites where specific transcription factors are required for lineage progression. (Adapted from Franceschi *et al.*, 2007.)

Transcriptional control of the osteoblast lineage

Once the positional identities of bone progenitor cells are defined by a unique combination of HD proteins, these transcription factors initiate the differentiation of NCC-derived mesenchymal stem cells to the major bone cell types by upregulating BMPs and other osteoinductive factors. On the basis of gene deletion studies, at least

six different transcription factors were identified as being necessary for various aspects of bone formation: SOX 5, 6, and 9 and Runx2 are required for chondrocyte differentiation, while Runx2, Osterix (OSX), and ATF4 are necessary for osteoblastogenesis. For a review, see Karsenty and Wagner (2002). The relationships between these factors during bone formation are summarized in Figure 6.2.

Runx2 is a commitment factor for the bone lineage. In this sense, it functions like other transcription factors such as SOX9, PPAR γ , and C/EBP or myoD/myogenins that control chondrocyte, adipocyte, and myoblast differentiation, respectively. Runx2 is first detected shortly after initial mesenchymal condensations in regions destined to form bone (embryonic day 10.5 in mice) and persists through the latter stages of development (Ducy *et al.* 1997). Runx2 is necessary for formation of both osteoblast and hypertrophic chondrocytes. Consequently, bone development in Runx2-null mice is arrested at the cartilage stage with no detectable differentiation of MSCs to osteoblasts or hypertrophic chondrocytes. As a result, skeletons of these mice have a relatively normal morphology but totally lack mineral. For this reason, neonates die of respiratory failure due to the inability of the rib cage to support breathing (Komori *et al.* 1997; Otto *et al.* 1997). Interestingly, tooth development in Runx2-null mice is also arrested before overt odontoblast and ameloblast formation in the developing tooth organ (D'Souza *et al.* 1999). Mutations in Runx2 are the basis for the human disease cleidocranial dysplasia (CCD), an autosomal dominant disorder characterized by hypoplastic clavicles, patent fontanelles of the skull, and supernumerary teeth (Mundlos *et al.* 1997; see also Chapter 7, this volume). Consistent with its role as a commitment factor for the bone lineage, ectopic overexpression of Runx2 in mesenchymal cells can induce bone formation in vivo (Yang *et al.* 2003). In fact, adenoviral and retroviral overexpression of Runx2 has been successfully used in several craniofacial regeneration models (Gersbach *et al.* 2004a; Zhao *et al.* 2005, 2007).

Runx2 is a member of the runt domain family of transcription factors, a group of molecules having broad functions in the development of the hematopoietic system (Runx1), bone (Runx2), and gut (Runx3). For a review, see Jensen (2007). All three have also been implicated in the etiology of certain cancers. Thus, Runx1, also known as AML1, is a common site for chromosomal translocations in acute myelogenous leukemias, while Runx2 is associated with highly metastatic cancers of the breast and prostate. In contrast, Runx3 may have a tumor suppressor function since it is downregulated in colon cancer (Pratap *et al.* 2006).

All runt-domain family members bind to the DNA of target genes via a specific enhancer with the consensus sequence, PuCCPuCA/T. One or multiple copies of this enhancer have been detected within regulatory regions of many bone-associated genes such as osteocalcin (Ducy *et al.* 1997), bone sialoprotein (Roca *et al.* 2005), osteopontin (Ducy *et al.* 1997; Sato *et al.* 1998), receptor activator of NF- κ B ligand (Kim *et al.* 2006), matrix

metalloproteinase 13 (Selvamurugan *et al.* 2000), and type X collagen (Dong *et al.* 2006). Runx2 forms complexes with a number of nuclear accessory factors including Cbfb, p300, DLX5, ATF4, histone deacetylases, and Smad proteins (mediators of Bmp and TGF β signaling; Kagoshima *et al.* 1996; Westendorf *et al.* 2002; Zaidi *et al.* 2002; Sierra *et al.* 2003; Roca *et al.* 2005; Xiao *et al.* 2005) and is also subject to a number of posttranslational modifications including phosphorylation, acetylation, and ubiquitination (Ge *et al.* 2009; Jonason *et al.* 2009). A specific domain in Runx2 tethers it to the nuclear matrix, a higher order nuclear structure that is responsible for the intranuclear localization of specific transcription complexes (Zeng *et al.* 1997). Of particular importance for craniofacial development, Runx2 binds and is inhibited by Twist1 and Twist2, two basic helix-loop-helix transcription factors previously associated with craniosynostoses (Bialek *et al.* 2004). During normal craniofacial bone development, Twist1/2 levels are elevated until just before suture closure when levels decline. As would be expected if TWIST were an inhibitor of bone formation, Twist1^{+/-} mice exhibit premature suture closure resulting in craniosynostosis. A similar situation is encountered in Saethre-Chotzen patients who are heterozygous for Twist1 deletions (el Ghouzzi *et al.* 1997). As further evidence for this inhibitory function, crossing Twist1^{+/-} mice with Runx2^{+/-} animals rescues the normally patent fontanelles of Runx2-null mice.

The zinc finger transcription factor, Osterix (OSX), was first identified as a BMP inducible protein in mesenchymal osteoprogenitor cells (Nakashima *et al.* 2002). Unlike Runx2-nulls, Osx knockout mice produce hypertrophic mineralized cartilage but still lack osteoblasts and mineralized trabecular or cortical bone. Since Runx2 is present at normal levels in Osx-null mice while OSX is absent in Runx2 knockouts, Osx is assumed to be functionally downstream from Runx2 and can be directly activated by Runx2 binding to its promoter (Nishio *et al.* 2006). In addition to being regulated by BMPs, Osx is stimulated by parathyroid hormone and this induction together with stimulation of ATF4 may explain, at least partially, the anabolic actions of this hormone (Yu *et al.* 2009).

ATF4 is an additional osteoblast-associated transcription factor having an important role in bone development. Unlike Runx2 and OSX, ATF4 is not essential for osteoblast differentiation. Instead, it modulates osteoblast activity once initial bone formation has occurred. Deletion of ATF4 in mice results in delayed bone formation during embryonic development and a low bone mass phenotype throughout life (Yang & Karsenty 2004; Yang *et al.* 2004). Unlike Runx2 and Osx genes that are

selectively expressed in skeletal tissues, ATF4 mRNA is present in most tissues but is selectively translated in cells like osteoblasts that secrete large amounts of proteins resulting in activation of the unfolded protein response. ATF4 is phosphorylated and activated by the growth factor–regulated kinase, RSK2, which is mutated in Coffin–Lowry syndrome, an X-linked disorder associated with mental retardation, delayed bone development, and short stature (Yang *et al.* 2004). ATF4 selectively binds to enhancer sequences in the regulatory regions of osteoblast-related genes like osteocalcin where it cooperates with Runx2 to stimulate transcription (Xiao *et al.* 2005). Significantly, ATF4-null mice have a blunted response to the anabolic actions of PTH indicating that this transcription factor can mediate some of the actions of this hormone (Yu *et al.* 2009).

Properties of mature osteoblasts and osteocytes

After commitment to the osteoblast lineage, mesenchymal stem cells undergo an orderly progression from periosteal preosteoblast cells adjacent to bone to secretory osteoblasts forming a monolayer on the bone surface (Figure 6.3). Osteoblasts are polarized cells

having a prominent endoplasmic reticulum and Golgi apparatus that allows them to secrete an extensive extracellular matrix (ECM). It is within this matrix that hydroxyapatite mineral is deposited to produce a mineral–protein composite material having the strength and elasticity necessary for skeletal integrity. The overall composition of bone by mass is approximately 65%–70% mineral, 10% water, and 20%–25% organic matrix. This matrix is predominantly composed of type I collagen (approximately 90%), with the balance composed of noncollagenous proteins and proteoglycans. For a review, see Robey and Boskey (2008).

Type I collagen, the prototypical fibrillar collagen, is a triple helical molecule containing two identical $\alpha 1(I)$ chains and a structurally similar, but genetically distinct, $\alpha 2(I)$ peptide. Individual collagen triple helices are arranged into fibrils with a characteristic quarter-staggered arrangement that gives the fibril its ultrastructural appearance. Fibrils also contain inter- and intrachain cross-links between adjacent lysine residues to provide further structural rigidity. In lamellar bone, hydroxyapatite mineral first appears in the gap zones separating adjacent collagen triple helices within fibrils and then propagates throughout the rest of the bone matrix (Christoffersen & Landis 1991). However, there

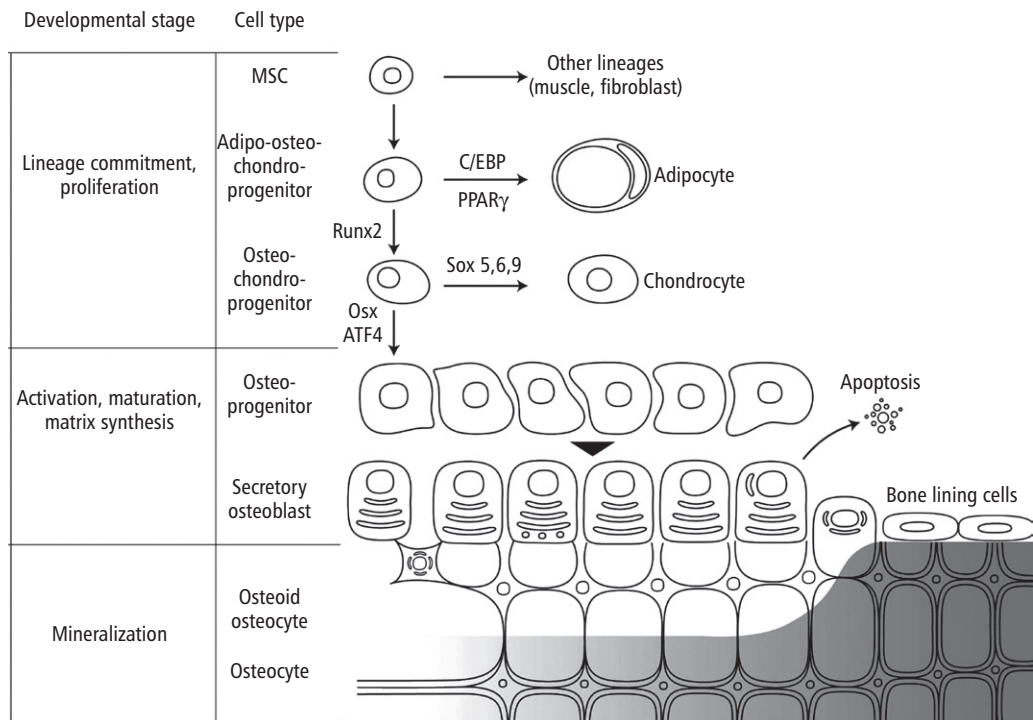


Figure 6.3 Relationship between mesenchymal stem cells (MSC), preosteoblasts, osteoblasts, and osteocytes on the bone surface. The right side of the figure shows the lineage of osteoblasts and osteocytes from pluripotent mesenchymal stem cells; the relationship between osteoprogenitors, osteoblasts, and osteocytes on the surface of bone; and the major transcription factors controlling lineage commitment decisions in this cell population. Mineralized matrix and entombed osteocytes are shown in gray.

is still some debate about the site of primary mineral nucleation, which may be associated with certain non-collagenous proteins in complex with vesicular structures (also called *matrix vesicles*) secreted by osteoblasts (Huffman *et al.* 2007).

The noncollagenous proteins of bone represent a broad class of molecules whose function remains largely undefined. Unlike collagen, which is widely distributed in connective tissues, several of the noncollagenous proteins are preferentially expressed in osteoblasts and/or osteocytes and are thought to give these cells the unique ability to produce a mineralized ECM. The best studied of these are osteocalcin, bone sialoprotein, dentin matrix protein 1, bone acidic glycoprotein 75 (BAG75), matrix extracellular phosphoglycoprotein (MEPE), and alkaline phosphatase (Tnap). It has been proposed that bone sialoprotein, DMP1, BAG75, MEPE, and alkaline phosphatase all participate in bone ECM mineralization. Bone sialoprotein can nucleate mineral under controlled in vitro conditions and is postulated to have a similar role in vivo in that Bsp-null mice have altered bone mineralization (Hunter & Goldberg 1993; Malaval *et al.* 2008). DMP1 can also nucleate mineral in vitro, and DMP1-null animals have severe defects in bone and tooth mineralization (Ling *et al.* 2005). However, results with DMP1 are complicated by systemic effects related to high levels of osteocyte FGF23 in DMP1 nulls that can induce systemic hypophosphatemia and secondary loss of bone mineral (Feng *et al.* 2006). BAG75 may exist as part of a vesicular complex that also contains bone sialoprotein. It is proposed that a proteolytic processing event is necessary to activate this complex and nucleate mineralization (Huffman *et al.* 2007). MEPE may be a negative regulator of mineralization as MEPE-null mice have increased bone density (Addison *et al.* 2008). MEPE's inhibitory function requires a proteolytic event catalyzed by PHEX protein (phosphate-regulating neutral endopeptidase on chromosome X) to generate the mineralization inhibitor, ASARM peptide (acidic serine- and aspartate-rich MEPE-associated motif). In contrast, the role of alkaline phosphatase in mineralization appears to be indirect via its ability to degrade pyrophosphate, which is, itself, a potent inhibitor of mineralization (Hessle *et al.* 2002; Addison *et al.* 2008). Osteocalcin's function in bone, if it has one, is related to suppression of mineralization in that deletion of the *Bglap* genes results in a mild elevation of bone mass (Ducy *et al.* 1996). More recently, however, osteocalcin has been shown to have a systemic role in regulating energy metabolism and insulin sensitivity (Ferron *et al.* 2008). Regardless of their function, bone sialoprotein (*Ibsp*) and osteocalcin (*Bglap1,2*) genes have been most useful as tools for understanding osteoblast-specific gene

expression and its regulation by growth factors and hormones.

Secretory osteoblasts have a limited in vivo lifetime estimated to be approximately three months in humans and only 10–20 days in mice (Franz-Odenaal *et al.* 2006). Once they stop secreting bone matrix, osteoblasts have three potential fates: (1) they can become embedded in bone as osteocytes, (2) they undergo apoptosis, or (3) they become inactive bone-lining cells. In human bone, it is estimated that only about 30% of osteoblasts become osteocytes (Manolagas 2000). The progression from preosteoblast to osteoblast and then to osteocyte was initially established by in vivo pulse/chase labeling (Owen 1963). More recently, real-time visualization of this process was achieved through the use of transgenic marking of osteoblasts and osteocytes using green fluorescent proteins emitting at different wavelengths. Individual transgenic mouse lines were developed expressing either green fluorescent protein (GFP) cyan under the control of a 2.3 kb *Col1A1* promoter to mark osteoblasts or GFP topaz under the control of an 8 kb DMP-1 promoter to mark osteocytes and preosteocytes. Using this approach, a clear transition of individual osteoblast cells into osteocytes could be visualized in calvaria (Dallas & Bonewald 2010).

Although clearly derived from osteoblasts, osteocytes have distinct phenotype and biological functions. For review, see Chapter 8 in this volume, Bonewald and Johnson (2008), and Franz-Odenaal *et al.* (2006). In contrast to the plump, polygonal morphology of osteoblasts, osteocytes gradually assume a more elongated shape with a smaller cell body and extensive dendritic processes as they become incorporated into the ECM. Early on, they continue to secrete matrix and may play an active role in the mineralization process (osteoid osteocytes). Mature osteocytes reside in lacunae within the mineralized ECM. Osteocytes communicate with each other and with osteoblasts on the bone surface through an extensive network of dendritic processes that use gap junctions to transfer signals between cells. Although they share some markers with osteoblasts (e.g., osteocalcin), they also uniquely express several proteins including DMP-1, MEPE, E11/gp38, PHEX, sclerostin, and FGF23. Because osteoblasts only line the surface of growing bone while osteocytes occupy the entire volume of bone matrix, this latter cell type vastly outnumbers the osteoblasts. It is estimated that 90%–95% of all bone cells are osteocytes while only 4%–6% are osteoblasts and 1%–2% are osteoclasts. In contrast to osteoblasts that have lifetimes of weeks to months, osteocytes can persist in the bone matrix for many years. In this sense, osteoblasts and osteoid osteocytes can be viewed as transitional cell types that secrete and mineralize bone ECM

for only a few weeks while mature osteocytes are the terminal cell in this lineage. Their long half-life and the fact that osteocytes are present regardless of whether or not active bone formation is taking place make them an ideal cell to sense changes in the bone microenvironment associated with fluctuations in circulating calcium and phosphate levels or altered mechanical loads.

Major regulatory functions of osteoblasts and osteocytes

In addition to being responsible for bone deposition, osteoblasts and osteocytes have extensive regulatory functions related to bone remodeling, phosphate homeostasis, and skeletal loading.

Bone remodeling

Although net bone growth ceases with the onset of adulthood, bone is constantly renewed by the concerted action of osteoclasts that resorb bone and osteoblasts that replace bone lost by resorption. Osteoblasts and osteoclasts form a recognizable anatomical structure known as the *basic multicellular unit* (BMU). Cells of the osteoblast lineage control both the formation and breakdown of bone necessary for remodeling. Bone formation and mineralization, as discussed in this chapter, is a primary function of the secretory osteoblast and related osteoid osteocyte. In contrast, participation of the osteoblast lineage in bone resorption is indirect via activation of osteoclasts. Osteoblasts respond to a variety of resorptive signals including the vitamin D hormone, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), parathyroid hormone (PTH), IL-1, IL-6, and sympathetic tone (β-adrenergic receptor activation) by secreting receptor activator of NF-κβ ligand (RANKL). RANKL, which, based on gene deletion studies, is indispensable for osteoclastogenesis (Hsu *et al.* 1999; Kong *et al.* 1999), functions by binding to its receptor, RANK. RANK activation stimulates a number of signal transduction pathways including IKK/IκB/NF-κβ, mitogen-activated protein kinase (MAPK), Src, and phosphatidylinositol 3-kinase (PI3K)/AKT, which activate c-fos, NF-κβ, and NFATc1 transcription factors to induce osteoclast differentiation from hematopoietic progenitors. For a review, see Teitelbaum and Ross (2003).

Because RANKL plays such a central role in osteoclastogenesis, a considerable effort has been devoted to understanding how it is controlled by PTH and 1,25(OH)₂D₃. The basis for this control was independently reported by two groups: Fu and colleagues (2006) and Kim and colleagues (2006). Interestingly, major regulation of the *Rankl* gene was localized to a distal region 76kb upstream from the transcription start site

called the RANKL distal control region (RL-DCR). The RL-DCR contains a functional vitamin D response element (VDRE), several glucocorticoid receptor response elements, a cAMP response element, and a binding site for Runx2. Deletion analysis established that the RL-DCR is critical for RANKL induction by PTH (via induction of cAMP and cAMP response element binding protein, or CREB) and 1,25(OH)₂D₃ and that responsiveness could be modulated by Runx2. Of further significance, deletion of the RL-DCR in mice reduced PTH and 1,25(OH)₂D₃ stimulation of RANKL mRNA and osteoclastogenesis in marrow cultures as well as RANKL mRNA induction in vivo. Furthermore, RL-DCR-null mice exhibited a high bone mass phenotype characterized by increased bone strength, reduced osteoclast and osteoblast numbers, and low bone turnover (Galli *et al.* 2008). Although RL-DCR deletion inhibited both 1,25(OH)₂D₃ and PTH responsiveness, residual 1,25(OH)₂D₃ induction of RANKL mRNA and osteoclastogenesis was still seen. This may be explained by other more proximal VDREs in the RANKL promoter that were also shown to bind vitamin D–retinoic acid receptor (VDR-RXR) complexes as measured by chromatin immunoprecipitation assays (Kim *et al.* 2006). Regulation of RANKL expression by 1,25(OH)₂D₃ is absolutely dependent on the VDR in that this regulation is not seen in VDR-null osteoblasts. However, VDR-null cells can still respond to PTH indicating that these two factors can function independently to control RANKL expression (Takeda *et al.* 1999).

It has been assumed for many years that RANKL is produced by osteoblasts or cells of the osteoblast lineage. Several lines of evidence support this notion: (1) osteoprogenitor cells (i.e., MSCs), osteoblasts, or osteocytes all produce RANKL, and co-culture of any of these cells with osteoclast precursors produces functional osteoclasts (for a review, see Suda *et al.*, 1999); (2) the RL-DCR of the RANKL gene contains a functional Runx2 binding site, and Runx2 present in cells of the osteoblast lineage can enhance cAMP induction of RANKL (Fu *et al.* 2006); and (3) Runx2 can physically interact with the VDR and stimulate its activity (Fu *et al.* 2006). However, it is still not clear which of these cells is the major RANKL-producing cell in bone capable of responding to 1,25(OH)₂D₃ and PTH. For review, see Corral and colleagues (1998) and Galli and colleagues (2009).

Regulation of phosphate homeostasis

The vitamin D endocrine system controls both calcium and phosphate levels by stimulating the uptake of these two ions in the gut and, together with PTH, by inducing osteoclast-mediated bone resorption. With PTH, it also stimulates renal tubular calcium reabsorption and

inhibits phosphate reabsorption. However, because PTH secretion by the parathyroids and, indirectly, formation of $1,25(\text{OH})_2\text{D}_3$ (via PTH stimulation of the 25-hydroxyvitamin D 1α -hydroxylase-CYP27B1) are controlled by serum calcium concentrations but not phosphate levels, these two endocrine factors are not able to directly control phosphate homeostasis. For many years, researchers sought an additional hormone that could respond directly to circulating phosphate concentrations. This phosphate-regulating hormone, or phosphatonin, is now known to be FGF23, a factor almost exclusively produced by osteoblasts and osteocytes (Feng *et al.* 2009). FGF23 is preferentially released from these cells when serum phosphate levels become elevated and in response to elevated $1,25(\text{OH})_2\text{D}_3$. FGF23 acts on several target tissues. Like PTH, it blocks renal tubular reabsorption of phosphate by inhibiting the renal phosphate transporters, Npt2a and Npt2c, and inhibits renal CYP27B1 expression to reduce $1,25(\text{OH})_2\text{D}_3$ synthesis and intestinal phosphate (and calcium) absorption (Larsson *et al.* 2004; Shimada *et al.* 2004a). Unlike PTH, FGF23 does not respond to changes in serum calcium and therefore provides a route for dealing with excess phosphate concentration when PTH is suppressed by normalization of serum calcium. Consistent with these observations, FGF23-null mice have hyperphosphatemia, moderate hypercalcemia, reduced serum PTH, and elevated $1,25(\text{OH})_2\text{D}_3$ (Shimada *et al.* 2004b; Sitara *et al.* 2006). FGF23 functions by binding to the FGF receptor, FGFR1(IIIc), together with a second protein, Klotho, previously associated with premature aging (Urakawa *et al.* 2006). Klotho-null mice have a similar phenotype to FGF23 nulls that is consistent with this factor being required for FGF23 signaling (Razzaque & Lanske 2006). Interestingly, the premature aging associated with Klotho deletions has been related to soft tissue calcification and elevated $1,25(\text{OH})_2\text{D}_3$ levels as would be expected with FGF23 resistance.

FGF23 secretion from osteoblasts or osteocytes is controlled by both serum phosphate and $1,25(\text{OH})_2\text{D}_3$ through mechanisms that are still not well understood. Osteocytes from DMP-1-null mice express high levels of FGF23. These mice have a similar hypophosphatemic phenotype to mice harboring mutations in the *PheX* gene. Similarly, humans with loss of function mutations in either *PHEX* or *DMP1* genes also have elevated FGF23 levels and hypophosphatemia. For a review, see Feng and colleagues (2009) and Quarles (2008). Taken together, these studies suggest that the osteocyte proteins, DMP-1 and *PHEX*, may be part of a common pathway to regulate FGF23 and serum phosphate. For a review, see Chapter 8 in this volume.

Response of bone to mechanical loads

Bone has the unique ability to alter its mechanical properties in response to the physical forces it experiences. For a review, see Robling and colleagues (2006). When subjected to a load (stress), bone deforms in proportion to the magnitude of the load. The amount of deformation is called *strain*. Under physiological loading, maximum strains are 2000–3000 μstrain (displacements of 0.2%–0.3%). When bone bends, one side is subjected to compression and the other to tension. Bone is able to modify its structure to minimize strain when exposed to dynamic, as opposed to static, loads (Lanyon & Rubin 1984). Strains exceeding a certain threshold will stimulate new bone formation to strengthen the bone and thereby reduce maximum strains to below the threshold. A corollary to this concept is that bone mass will be lost with disuse when bone is subject to minimal strain (Frost 1988). There are many common examples illustrating how bone mass alters in response to loading or its absence. For example, the serving arm of professional tennis players has more bone mass than the nonserving arm (Krahl *et al.* 1994). In contrast, skeletal unloading during bed rest is associated with severe bone loss of up to 2% per month, while weight-bearing exercise increases bone mass (Gleeson *et al.* 1990; Leblanc *et al.* 1990).

There are two main ways bone cells might detect strain in response to mechanical loads: (1) direct detection of strain to individual cells as transmitted by cell contacts with the extracellular matrix, or (2) detection of fluid flow through bone canaliculi in response to strain. This latter response is initiated by the compression and tension across the bone during loading, which squeezes interstitial fluid through canaliculi and through the marrow cavity. These two pathways are not mutually exclusive, and both types of responses have been detected. Although no definitive experiments have excluded one or the other of these possibilities, fluid flow is thought to be the predominant way in which bone cells sense loads. Marrow stromal cells, including mesenchymal stem cells, osteoblasts, and osteocytes, can all respond to fluid flow shear stress (FFSS) *in vitro*. Although the positioning of osteocytes within canaliculi allows them to be exposed to the most consistent load-induced fluid flow changes, current evidence is equivocal concerning their exclusive role as mechanosensory cells. When osteocytes are selectively ablated in bone by targeted expression of diphtheria toxin using the DMP-1 promoter, bones are resistant to unloading-induced bone loss, suggesting that osteocytes are required for mechanotransduction (Tatsumi *et al.* 2007). This approach, which killed 70%–80% of osteocytes, left osteoblasts largely intact, yet prevented the induction of RANKL that normally

accompanies unloading. The response of animals to mechanical stimulation was not reported. However, reloading of tail-suspended mice was still able to stimulate new bone formation regardless of whether or not osteocytes had previously been ablated, suggesting that they are not required for this component of the response to loading.

FFSS stimulates a number of primary and secondary responses in target cells including stimulation of L-type calcium channels (Rawlinson *et al.* 1996), nitric oxide synthase (Pitsillides *et al.* 1995; Fan *et al.* 2004), cyclooxygenase and prostaglandin synthesis (Jee *et al.* 1990), Wnt protein secretion, and downregulation of the Wnt inhibitor, sclerostin (Arnsdorf *et al.* 2009; Lin *et al.* 2009). In addition, FFSS stimulates integrin-mediated activation of focal adhesion kinase (FAK; Young *et al.* 2009) and stimulation of several signal transduction pathways including the ERK, p38, JNK MAPK, and PI3K/AKT pathways (Boutahar *et al.* 2004; Sen *et al.* 2009). The end result is to stimulate mesenchymal stem cell recruitment and osteoblast differentiation leading to new bone formation (Li *et al.* 2004).

The responsiveness of bone to mechanical stimulation is modified by several humoral factors including PTH (Ma *et al.* 1999), estrogens (Damien *et al.* 1998; Lee *et al.* 2003), and insulin-like growth factors (Gross *et al.* 2002). In many cases, the effects of hormones on mechanoresponsiveness may be related to modulation of common pathways regulated by both signals. For example, modulation of mechanoresponsiveness by estrogens may be related to the postulated nongenomic effects of estrogen receptors α and β (ER α , ER β) on the ERK–MAP kinase pathway (Plotkin *et al.* 2005; Aguirre *et al.* 2007). Both ERs are required for maximal activation of ERK by mechanical stimulation of osteoblasts or osteocytes (Aguirre *et al.* 2007). In addition, nuclear localization of receptors is not required in that ERK activation can be achieved with ERs lacking a DNA binding domain. Rather, a subfraction of ER can function in association with caveolin-1 on the plasma membrane consistent with a nonnuclear function.

Signaling pathways affecting osteoblast function

Osteoblasts receive signals from a wide range of factors including mechanical loads, hormones, growth factors, and morphogens. After binding to the appropriate receptors, these factors activate a number of signal transduction pathways to control cellular activity. Two of the most widely used pathways are mitogen-activated protein kinase pathways and Wnt. Both pathways are necessary for responsiveness to hormones and growth factors, and

extracellular matrix and mechanical cues, and, in many cases, can affect osteoblast differentiation or function.

MAPK pathways

Three related mitogen-activated protein kinase (MAPK) pathways have been described in mammals: the ERK/MAPK, p38, and Janus kinase (JNK) pathways. All three are active in osteoblasts, although the former two have been most extensively studied.

The canonical ERK–MAPK pathway is activated by multiple signals encountered by osteoblasts and osteocytes including those initiated by growth factor receptors such as receptors for insulin, IGF-1, fibroblast growth factor, and BMPs (Cobb *et al.* 1991); extracellular matrix and integrin binding and FAK activation (Franceschi & Xiao 2003); related biomechanical stimulation (Moalli *et al.* 2001); and certain nongenomic actions of estrogens (Kousteni *et al.* 2003). Regardless of the initiating stimulus, downstream signals involve activation of Raf kinases that phosphorylate and activate the dual-specificity kinases, MEK1 and MEK2. MEKs subsequently phosphorylate or activate ERK1 and ERK2 to stimulate gene expression by phosphorylating specific transcription factors (Robinson & Cobb 1997). There is compelling *in vivo* evidence that ERK–MAPK signaling is critical for osteoblast differentiation. As shown by Ge and colleagues (2007), sustained transgenic overexpression of constitutively active (Mek-sp) or dominant negative MEK1 (Mek-dn) in osteoblasts, respectively, increased or decreased skeletal maturation during mouse development. Furthermore, crossing TgMek-sp mice with Runx2^{+/-} animals enabled the partial rescue of the CCD phenotype caused by Runx2 deficiency, while TgMek-dn, Runx2^{+/-} mice died at birth with a more severe CCD, which was consistent with the concept that MAPK activates the limiting amounts of Runx2 in heterozygous animals. In addition, selective inactivation of ERK2 in mesenchymal cells (osteoblast and chondrocyte progenitors) of ERK1-null mice using a Prx1-Cre was shown to cause severe defects in endochondral and intramembranous bone formation, decreased osteoblast differentiation, and ectopic cartilage formation (Matsushita *et al.* 2009). These studies provide conclusive proof that the ERK–MAPK pathway stimulates osteoblast differentiation and inhibits chondrogenesis *in vivo*.

In the case of osteoblasts, a major ERK substrate is Runx2, an essential transcription factor for the osteoblast lineage (Ge *et al.* 2009). ERK binds to Runx2 via a specific docking site in the runt domain and directly phosphorylates two critical residues at S301 and S319 (mouse sequence, type II Runx2 isoform) as measured by mass spectroscopy and biochemical assays. Serine to alanine mutation of these sites blocks the ability of

Runx2 to stimulate osteoblast-specific gene expression and differentiation. Interestingly, the ERK–Runx2 interaction was shown to occur on the chromatin of target genes and is essential for subsequent stimulation of gene expression (Li *et al.* 2010). The ERK–MAPK pathway can also affect osteoblast differentiation indirectly by activating the ERK1/2 substrate, RSK2, which subsequently phosphorylates ATF4, another critical factor for osteoblast differentiation (Yang *et al.* 2004). Mutations in RSK2 cause Coffin–Lowry syndrome, an X-linked disorder associated with mental retardation and skeletal anomalies (Yang *et al.* 2004).

The stress- and cytokine-activated MAPK, p38 MAPK, may also participate in RUNX2 activation and osteoblast differentiation, likely via a mechanism similar to that described here for ERK–MAPK (Greenblatt *et al.* 2010). In addition to signaling through SMAD proteins, TGF β and BMP receptors activate TAK1 (TGF β -activated kinase), which stimulates ERK, p38, and JNK MAPK pathways (Shim *et al.* 2009). Osteoblast-selective deletion of TAK1 in mice causes severe defects in endochondral and intramembranous bone formation. Furthermore, deletion of the p38 pathway intermediates Mkk3, Mkk6, p38 α , or p38 β also leads to reduced bone mass and defective osteoblast differentiation, suggesting that at least some of the effects of TAK1 deletion can be attributed to defects in p38 signaling. Furthermore, p38 α and p38 β can phosphorylate Runx2. Mass spectroscopy revealed that one of the sites phosphorylated by ERK (S319) is also a possible p38 substrate as well as additional sites at S28 and S250. Combined mutation of S28, S250, and S319 significantly reduced the ability of p38 to stimulate a Runx2 reporter gene. Studies on ERK and p38 MAPK signaling in osteoblasts raise the intriguing possibility that Runx2, via its phosphorylation state, may be able to integrate diverse signals from hormone or growth factor receptors, TGF β /BMP receptors, integrins, and other mechanoreceptors to control the overall level of osteoblast gene expression.

The Wnt pathway and control of osteoblast versus adipocyte lineages

Bone marrow contains mesenchymal stem cells capable of differentiating to osteoblasts or adipocytes. These cells have been a focus of considerable interest because of the well-known property of marrow to accumulate fat with aging and the association of marrow adiposity with reduced bone mass and osteoporosis (Meunier *et al.* 1971; McDonough *et al.* 2008). The Wnt pathway controls cell migration, proliferation, and differentiation during tissue development as well as in certain cancers and can preferentially stimulate the lineage of marrow MSCs toward osteoblasts at the expense of adipocytes.

In the absence of Wnt ligands, Wnt target genes are quiescent because of β -catenin phosphorylation and degradation catalyzed by a complex containing glycogen synthase kinase 3 β (GSK3 β), adenomatous polyposis coli (APC), and axin. The pathway is activated by binding of Wnt ligands to frizzled receptors and low-density lipoprotein receptor-related protein 5 and 6 (LRP5,6) co-receptors. This inactivates GSK3 β via phosphorylation, leading to disruption of the degradation complex and release of β -catenin. Active β -catenin then translocates to the nucleus where it interacts with T cell factor–lymphoid enhancer binding factor (TCF–LEF) to stimulate the transcription of target genes. Wnt signaling can also be modulated by the inhibitors, secreted frizzled-related protein (SFRP2) and Dickkopf proteins (DKKs), which inhibit Wnt–LRP receptor interactions (Bodine *et al.* 2004; Kawano & Kypta 2003). In bone, the Wnt signaling pathway is essential for osteoblastogenesis and osteogenesis (Gong *et al.* 2001). Many of the components of this pathway have been characterized by gain-of-function or loss-of-function mutations in mice. These studies established an important role for Wnt signaling in bone formation. For example, in conditional deletion of β -catenin in early osteo-chondroprogenitors or in Runx2⁺, Osx⁺ progenitors blocked formation of mature osteoblasts and diverted the cell lineage to chondrocytes. In contrast, ectopic β -catenin expression enhanced osteoblast differentiation (Hill *et al.* 2005; Rodda & McMahon 2006). Consistent with these findings, transgenic overexpression of Wnt10b in the adipogenic cells of bone marrow increased bone mass presumably by stimulating resident marrow MSCs to progress down the osteoblast lineage (Bennett *et al.* 2005). In contrast, DKK1 haploinsufficiency increased bone formation, while DKK1 overexpression in calvarial osteoblasts stimulated adipocyte differentiation (Glinka *et al.* 1998; Morvan *et al.* 2006).

Use of osteoblasts and osteoprogenitors for cranial bone regeneration

Intrinsic mechanisms of bone repair

Bone has an intrinsic but finite capacity to heal following fracture or resection and during distraction osteogenesis. The mechanisms regulating bone repair are not well understood, but it is clear that a variety of soluble factors, extracellular matrix (ECM) proteins, and cell types participate in a sequential process having the following steps: (1) inflammation and hematoma, (2) cartilage formation, (3) cartilage resorption coupled with bone formation, and (4) remodeling of the new bone (Gerstenfeld *et al.* 2003; Alford & Hankenson 2006; Ai-Aql *et al.* 2008). Cytokines of the interleukin family, the tumor necrosis

factor- α family, and morphogens including TGF β 1, BMP2, and FGF2 are upregulated in the acute response to injury and drive the recruitment of mesenchymal progenitors. The marrow, endosteum, and periosteum are primary sources of the progenitor cells that participate in bone healing (Colnot 2009). Significantly, the proliferation and osteogenic potentials of mesenchymal progenitors from neural crest-derived bone (e.g., mandible) are quantitatively different from those from mesoderm-derived bone (e.g., tibia; Leucht *et al.* 2008). TGF β superfamily morphogens (e.g., BMP2) promote the differentiation of progenitors into osteoblasts and chondrocytes, which mediate intramembranous ossification at the periosteal margins of the fracture and formation of cartilage in the “soft” callus (Dimitriou *et al.* 2005). In addition to TGF β s and BMPs, Wnts regulate osteoblast activity in regenerating bone by promoting the proliferation of mesenchymal progenitors (Secretò *et al.* 2009). Canonical Wnt signaling, mediated by β -catenin, is required for expression of Runx2 in osteoblasts (Kim *et al.* 2007a), and fractures in mice sensitized to Wnts through inactivation of the negative Wnt regulator Axin2 exhibited faster bone repair than wild-type controls (Minear *et al.* 2010). Following deposition of cartilage in the soft callus, the regenerating bone begins to mineralize via an endochondral ossification process similar to that observed during limb development. Angiogenic factors, including VEGFs and angiopoietins, promote vascular invasion of the callus, apoptosis of the chondrocytes, and resorption of the cartilage matrix. Osteoblasts are an important source of these factors, as activation of the HIF-1 α pathway in osteoblasts upregulates VEGF and accelerates regeneration (Wan *et al.* 2008). In the later stages of regeneration, perivascular mesenchymal progenitors migrate into the callus, differentiate into osteoblasts, and assemble a primary mineralized bone matrix. The repair process concludes as the new bone undergoes remodeling by osteoclasts and osteoblasts during a secondary bone formation stage.

Mechanical loading of the regenerating bone (e.g., in an unstabilized fracture) promotes callus formation (McKibbin 1978) and appears to be essential for differentiation of osteoblasts within the regenerate (Leucht *et al.* 2007) much as it is during normal development (discussed in this chapter). Osteoblasts lacking FAK, a critical integrin-signaling and cytoskeletal protein, are capable of differentiating but fail to organize ECM and osteoid resulting in delayed healing (Kim *et al.* 2007b). In addition, the BMP signaling axis is sensitive to mechanical loading. BMPs, BMP receptors, and Smad phosphorylation are upregulated in an unstabilized fracture and during distraction osteogenesis of the mandible in comparison to stabilized fractures and static controls

(Khanal *et al.* 2008; Yu *et al.* 2010). Thus, the recruitment, proliferation, and differentiation of osteoblasts are regulated by a variety of mechanical and chemical cues throughout the intrinsic bone-healing process.

Strategies for enhancing regeneration of bone

The current standard of care for bridging large bony defects (e.g., after trauma or cancer resection or to correct genetic defects like cleft palate) is transplantation of autologous tissue (Pollak & Ficke 2008; Zuk 2008). However, the short supply of autologous bone and complications with donor site morbidity limit the utility of this approach and have motivated the research and development of ex vivo engineered tissues and biologic (i.e., cell-, protein-, or gene-based) therapies that augment the body's intrinsic regenerative responses. One substantial challenge in the development of engineered bone is generating enough osteoblasts and supporting cells to form de novo a large, load-bearing mineralized matrix. A variety of relatively abundant cell types, including skeletal myoblasts, dermal fibroblasts, and marrow-derived stromal cells, can adopt an osteoblastic phenotype upon transduction with a Runx2 expression vector (Gersbach *et al.* 2004b; Zhao *et al.* 2005, 2007; Byers *et al.* 2006; Phillips *et al.* 2007; Wojtowicz *et al.* 2010). Similarly, overexpression of BMP2 and/or BMP7 in fibroblasts dramatically increases the in vitro and in vivo expression of osteoblast markers (Koh *et al.* 2008; Krebsbach *et al.* 2000; Rutherford *et al.* 2002). Co-delivery of angiogenic and osteogenic factors, such as VEGF and BMP2, can further enhance bone deposition in engineered bone and calvarial defects (Peng *et al.* 2002; Huang *et al.* 2005; Patel *et al.* 2008; Young *et al.* 2009), although the relative doses and timing of growth factor delivery have yet to be optimized. Exogenous activation of the Wnt pathways via recombinant Wnts (Minear *et al.* 2010; Popelut *et al.* 2010) or pharmaceutical antagonists of the PPAR γ pathway (Krause *et al.* 2010) is also effective in promoting osteoblast differentiation and regeneration of bone.

Guiding principles for the design of bone tissue constructs are now emerging, as the osteoblastic differentiation of mesenchymal progenitors has been shown to be sensitive to the mechanical properties (Engler *et al.* 2006; Khatiwala *et al.* 2006, 2009; Huebsch *et al.* 2010), topographic features (Olivares-Navarrete *et al.* 2008), and surface chemistry (Keselowsky *et al.* 2005; Reyes *et al.* 2007; Petrie *et al.* 2010) of the substrate. Moreover, complex tissue-level geometries of craniofacial bones can now be recapitulated with high fidelity using clinical imaging modalities, computer-aided design, and solid free-form fabrication of degradable scaffolds (Hollister

et al. 2005). These synthetic scaffolds support osteoblast adhesion and differentiation in vivo and serve a load-bearing and space-filling function until the new bone is synthesized and remodeled. Mechanical signals such as fluid shear stress and the enhanced chemical transport associated with fluid flow promote osteoblast differentiation, matrix deposition, and mineralization, particularly in three-dimensional bone tissue constructs (Sikavitsas *et al.* 2003; van den Dolder *et al.* 2003; Datta *et al.* 2006; Grayson *et al.* 2010). Cell-to-cell interactions are also important for enhancing bone formation and maintaining the viability of osteoblasts in three-dimensional (3D) culture. Overexpression of the gap junction protein connexin 43 increased the abundance and homogenized the spatial distribution of mineral in an MSC-populated bone construct (Rossello *et al.* 2009), and the co-culture of dental epithelial and mesenchymal cells was required for the formation of calcified nodules in engineered teeth (Zhang *et al.* 2010). Taken together, these studies reveal a spectrum of biologic pathways available for guiding the osteoblast-mediated formation of new bone ex vivo and the regeneration of large bony defects in vivo.

Summary and future directions

Osteoblasts in the craniofacial region are derived from the neural crest. Positional signals embedded in the expression patterns of homeodomain transcription factors and gradients of diffusible molecules such as endothelins, hedgehogs, fibroblast growth factors, and bone morphogenetic proteins specify sites for osteoblast differentiation and bone formation. Osteoblasts are derived from mesenchymal stem cell (MSC) progenitors and are only transiently present during periods of active bone formation when their differentiation from MSCs is stimulated. This differentiation program is controlled by several transcription factors including Runx2, Osterix, ATF4, and a number of accessory factors necessary for expression of the osteoblast phenotype. The primary function of osteoblasts is to secrete and mineralize the type I collagen-containing extracellular matrix of bone. The mineralization process is not well understood, but is likely mediated by several noncollagenous proteins that induce or inhibit mineralization. A fraction of osteoblasts become entrapped in the matrix they secrete as osteocytes, a long-lived cellular component of bone having a life span measured in years. Osteocytes do not secrete large amounts of ECM proteins, but may participate in the mineralization process that occurs in bone osteoid distal to the osteoblast layer. Cells of the osteoblast lineage also have a number of regulatory functions including control of bone remodeling, regulation of

phosphate homeostasis, and response to mechanical loads. They carry out these functions in response to specific humoral factors like parathyroid hormone, 1,25-dihydroxyvitamin D₃, FGF23, and local biomechanical forces, which activate specific signal transduction pathways to control osteoblast activity. Recent studies have begun to exploit basic science knowledge concerning control of osteoblast differentiation and activity to regenerate craniofacial bones. This is likely to be an important goal for future translational work in this area and has the potential to eventually correct bone lesions caused by developmental anomalies, surgical interventions, or trauma.

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Clinical correlate: cleidocranial dysplasia

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Bone formation is regulated by osteoblasts. Osteoblasts originate from mesenchymal cells that possess the potential ability to differentiate into several different lineages. Osteoblastic differentiation is regulated by various transcription factors. Of these, Runx2 is a master regulator of osteoblastogenesis: Runx2-deficient (Runx2^{-/-}) mice completely lack osteoblasts (Otto *et al.* 1997), and the forced expression of Runx2 in fibroblasts converts them so that they possess some of the characteristics of osteoblasts: for example, they begin to express osteoblast-specific markers, such as osteocalcin and bone sialoprotein (Ducy *et al.* 1997). Accordingly, Runx2^{+/-} mice display hypoplasia of the clavicles and open fontanelles, which resembles human cleidocranial dysplasia (CCD; Komori *et al.* 1997).

CCD (MIM #119600) is a skeletal dysplasia characterized by persistently open sutures or delayed closure of sutures, wormian bones, frontal bossing, hypoplastic or aplastic clavicles, short stature, wide pubic symphyses, delayed eruption of the permanent dentition, supernumerary teeth, and other skeletal abnormalities affecting the entire skeleton (Mundlos 1999). It is a rare disease, and its incidence is estimated as 1:1,000,000. The condition is inherited as an autosomal dominant trait; however, most cases are sporadic (Mundlos 1999). Advances in molecular genetics have revealed that the genetic cause of CCD is a mutation in the Runx2 gene (Mundlos *et al.* 1997). Indeed, hundreds of mutations in the Runx2 gene have been identified thus far in CCD patients (Otto *et al.* 2002). Three CCD cases are presented in this chapter that demonstrate different disease manifestations at different stages of development.

Case reports

Case 1: a two-year-old girl

A seven-month-old girl was brought to an orthopedic specialist at the hospital because of a widely dilated anterior fontanelle, but no definitive diagnosis was made, and she was left untreated. At two years of age, she was again brought to the orthopedic specialist. At this time, a whole-body X-ray survey was performed to search for abnormal skeletal development. The X-rays showed that she had multiple features characteristic of CCD: her cranial sutures were not closed (Figure 7.1A), and the anterior fontanelle was open. Her right clavicle was not properly joined but rather separated into pieces (Figure 7.1B), and the left clavicle showed malformation. She also had a cone-shaped thorax. Moreover, hypoplasia of the iliac bones and an unossified symphysis pubis and coxa valga were observed when she reached 10 years of age (Figure 7.1C).

Case 2: newborn with a severe manifestation

A two-month-old boy was referred to the hospital to obtain a second opinion for CCD. He developed normally in utero, and he was delivered vaginally without complications at 41 weeks gestation with a birth weight of 2430 g. At birth, because of severe hypoplasia of the parietal bones, a large anterior fontanelle was connected to the posterior fontanelle (Figure 7.2A), suggesting cranial dysplasia. This led the attending physician to search for abnormalities in the clavicles. The clavicles were not observed on palpation (Figure 7.2B), which enabled the physician to physically attach both

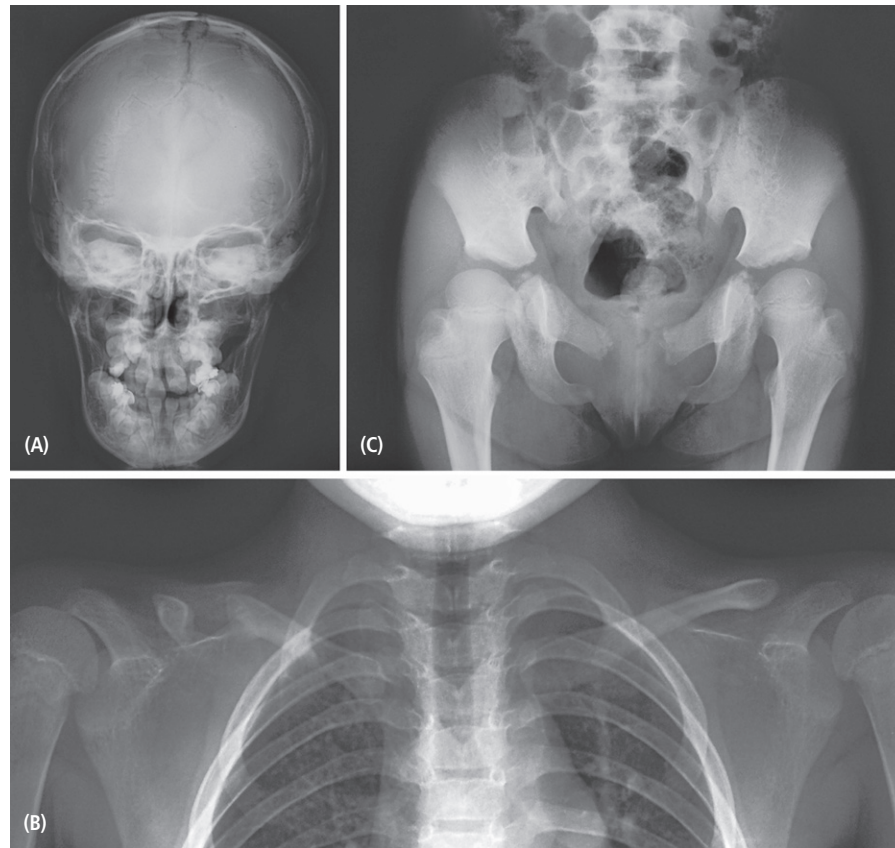


Figure 7.1 Radiographic analysis of the skeleton of the 10-year-old girl seen in Case 1. Radiographs of the (A) skull, (B) chest, and (C) pelvis.

shoulders, a hallmark of CCD. No abnormality in tooth development was observed. A molecular diagnostic exam was performed after obtaining consent from the patient's parents; the exam revealed the C631T mutation corresponding to the R211W missense mutation in exon3.

Case 3: an adult with supernumerary tooth formation

This male patient reported a wide-open fontanelle noted at one month of age. Thereafter, he visited his family doctor annually for checkups of his skeletal development. At 10 years of age, there was concern about his patent fontanelles. He was diagnosed with CCD because of the abnormalities in his skull and clavicles. Surgical closure of the anterior fontanelle was performed using artificial bones (Figure 7.3A). At the same time, impacted teeth and a delay in the eruption of the permanent teeth were documented but left untreated. At the age of 25 years, the patient visited an orthognathic specialist seeking treatment for his misaligned teeth (Figure 7.3B). He presented with reversed occlusion, and a pantomographic view of the permanent dentition revealed mul-

tipale, unerupted supernumerary teeth (Figure 7.3C). A diagnosis of malocclusion due to CCD was made.

Discussion

These three cases of CCD have different clinical presentations. Case 1 follows the typical disease manifestation. CCD was suspected at an age of several months, and a definitive diagnosis was provided at two years of age when a "patent" anterior fontanelle was confirmed at the age that fontanelles are expected to close completely in normal development. The patient also presented hallmarks of CCD: abnormal shape of the thorax, malformation of the clavicle, and delay in pelvic bone development. In Case 2, because of the severe abnormality in the skull and the clavicles, CCD was clinically diagnosed at birth, followed by molecular confirmation. In Case 3, as CCD did not affect the patient's intellect or motor development, he was left untreated until he was 10 years old, at which time treatment was requested for the patent anterior fontanelle. His misaligned teeth were corrected at a later date.

In a patient with accompanying patent fontanelles, hypoplastic clavicles, and supernumerary teeth, the defi-

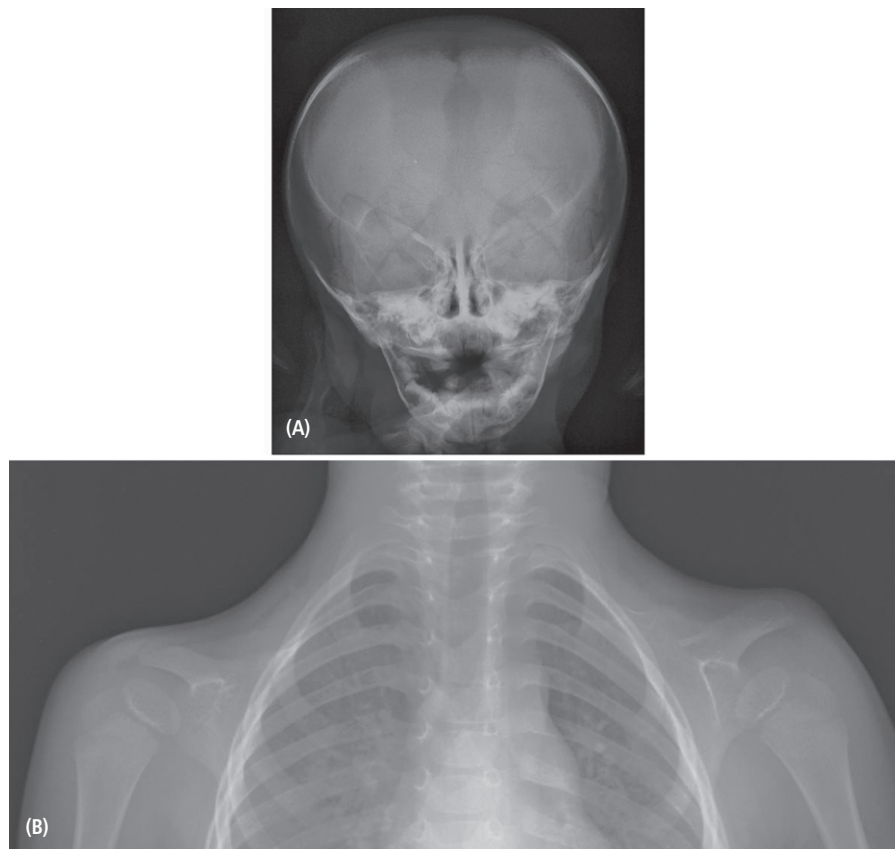


Figure 7.2 Radiographic analysis of the skeleton of the 10-year-old boy seen in Case 2. (A) Skull radiograph taken at one year of age; and (B) chest radiograph taken at five years of age. Note the large fontanelle and absence of clavicle.

nitive diagnosis is not difficult. However, quite often some of the symptoms are missing (Mundlos 1999). Moreover, phenotypic manifestation varies, even within the same family carrying identical mutations (Mundlos 1999). Dental abnormalities often accompany CCD; in fact, they are a characteristic of it. Deciduous tooth retention and a subsequent delay in the eruption of the permanent teeth are common (Suda *et al.* 2010). Patients sometimes endure living with no teeth because of the delay in the eruption of permanent teeth. These patients require surgical extraction of deciduous teeth and removal of bones superior to the permanent teeth to promote eruption. The other dental characteristic of CCD is supernumerary teeth. The number of supernumerary teeth sometimes reaches 70 (Suda *et al.* 2010). Interestingly, while Runx2^{+/-} mice have no overt abnormality in tooth development, human counterparts with CCD do display abnormalities in tooth development (Zou *et al.* 2003), suggesting that the role of Runx2 in tooth development differs in mice and humans. Moreover, the appearance of supernumerary teeth considerably varies among patients and even among siblings

possessing identical mutations of Runx2 (Suda *et al.* 2010). Thus, the pathogenesis of supernumerary teeth in CCD patients includes not only genetic mutation, but also other causes such as environmental effects.

Screening of the Runx2 gene in CCD patients does not always identify mutations. In fact, only approximately two-thirds of patients presenting with CCD have mutations in the coding region of Runx2 (Otto *et al.* 2002); the remaining third possess mutations in other genes such as MSX2 or core-binding factor beta (CBFB), a heterodimer partner of Runx2. Mutations within the promoter of Runx2 have also been reported. In addition, heterozygous deletions or duplications affecting the Runx2 gene have been identified in approximately 10% of CCD patients, which corresponds to the 25% of patients diagnosed as normal through sequence analysis (Zou *et al.* 2003). However, there are still some patients presenting with CCD whose genetic cause cannot be identified, even with intense study. Thus, other genes involved in the Runx2 signaling cascade may be involved in the pathogenesis of CCD, though no locus heterogeneity of CCD has been shown.

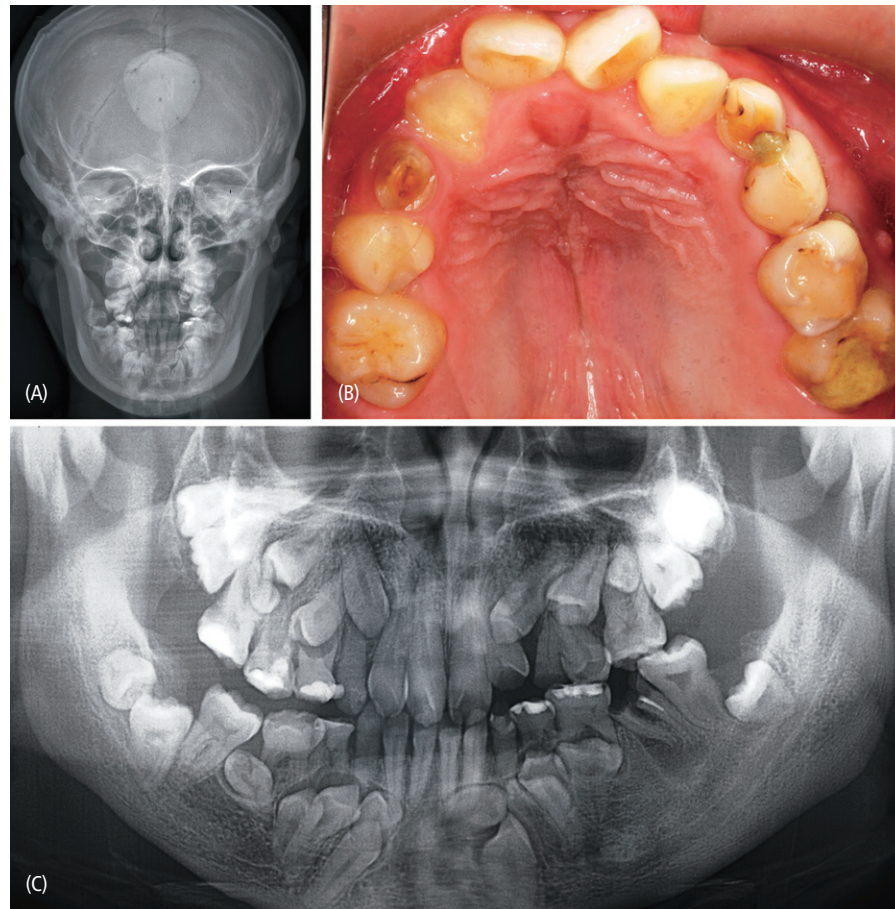


Figure 7.3 Analysis of the patient in Case 3 at 25 years of age. (A) Skull radiograph, (B) intraoral photograph, and (C) panoramic radiograph.

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Cell biology of craniofacial bone: osteocytes

Lynda F. Bonewald

Osteocytes compose 90%–95% of all bone cells in the adult skeleton. In the growing postnatal skeleton, the bone surface is covered by osteoblasts and osteoclasts modeling bone. However, in the adult skeleton, the majority of cells covering the bone surface are lining cells with 2%–5% osteoblasts and even fewer osteoclasts. Though osteocytes are descended from osteoblasts, a dramatic transformation takes place as the polygonal osteoblast begins to generate dendritic processes, and during the embedding process loses almost two thirds of its cytoplasm (Figure 8.1). It has been proposed that these cells regulate the mineralization process while embedding in osteoid (Barragan-Adjemian *et al.* 2006). This is evident by the expression of molecules known to regulate mineralization that include phosphate-regulating neutral endopeptidase on Chromosome X (PheX) and dentin matrix protein 1 (DMP1) (discussed in this chapter). These cells continue to be connected to each other; to cells on the bone surface such as lining cells, osteoblasts, osteoclasts, and their precursors; and to cells of the vasculature such as vascular epithelial cells by dendritic processes (Figure 8.2). Once embedded in mineralized bone, the cells reside with “caves” called *lacunae* (10–20 μ m) and send their dendritic processes through “tunnels” called *canaliculi* (200–300 nm), and they are continuously bathed by a bone fluid of unknown composition.

Recently, considerable interest has been generated concerning osteocyte function. These cells appear to be multifunctional and act as endocrine cells. The proposed functions of osteocytes include (1) mechanosensory cells, (2) regulators of osteoclast recruitment, (3) inhibitors of osteoblastic bone formation, (4) orchestrators of

bone remodeling, (5) regulators of phosphate homeostasis, (6) regulators of calcium homeostasis, and (7) endocrine cells.

Not only do DMP1 and PheX play a role in mineralization, but also these proteins downregulate the expression of fibroblast growth factor 23 (FGF23) that targets the kidney. FGF23 allows the reabsorption of phosphate by the kidneys to insure sufficient circulating phosphate to maintain normal bone mineral content. Since FGF23 is produced by osteocytes, these cells function as an endocrine organ to affect kidney function. DMP1 null mice have a similar phenotype to hyp mice carrying a PheX mutation (i.e., osteomalacia and rickets due to elevated FGF23 levels in osteocytes) (Feng *et al.* 2006). Autosomal recessive hypophosphatemic rickets in patients is due to mutations in DMP1 (Feng *et al.* 2006). Mutations in PheX are responsible for autosomal dominant hypophosphatemic rickets (Bonewald 2007b).

Additional functions have been proposed for osteocytes. It has been shown that osteocytes play a role in calcium homeostasis (Powell *et al.* 2011) and can regulate calcium availability by removing and replacing their mineralized matrix under normal, healthy conditions such as lactation (Qing in press). Osteocytes appear to be orchestrators of bone formation and resorption in addition to playing a role in mineral homeostasis. Osteocytes produce a factor, sclerostin, that inhibits osteoblastic bone formation (Paszty *et al.* 2010). This molecule, now the target of therapeutics as an antibody to sclerostin, has been shown to enhance bone mass and promote bone healing. Conversely, osteocytes can support osteoclast formation, especially in their dying or apoptotic state (Noble *et al.* 2003; Tatsumi *et al.* 2007). Osteocytes

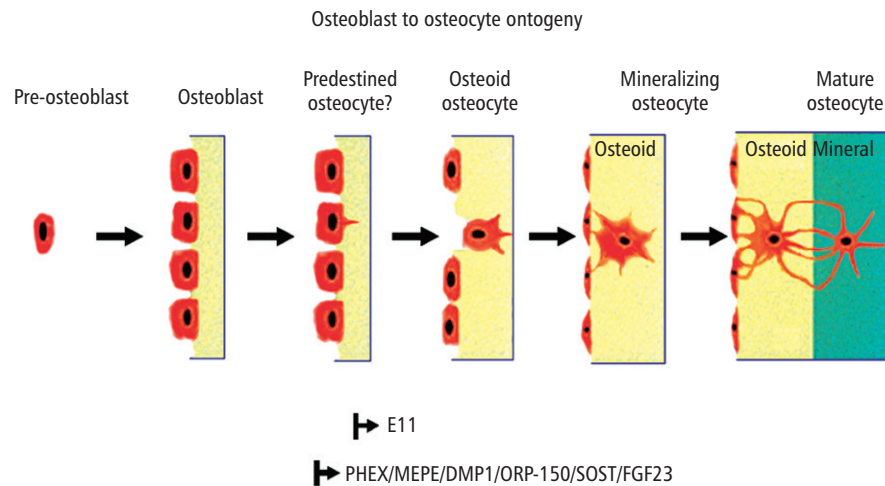


Figure 8.1 Osteoblast-to-osteocyte differentiation. The preosteoblast differentiates into the polygonal osteoblasts that function mainly to produce osteoid or unmineralized bone matrix. There are many well-known markers expressed during osteoblast differentiation such as osterix, runx2, alkaline phosphatase, collagen type II, and others (not shown in the figure). Osteocalcin (not shown in the figure) and Phex are produced by the late osteoblast and continue to be expressed by the osteocyte. For a full review on osteoblast-to-osteocyte differentiation, see Bonewald (2007a, 2011). By an unknown mechanism, some designated cells begin to embed in osteoid and begin to extend dendritic projections to existing cells in the bone matrix. Molecules such as E11/gp38 and MT1-MMP appear to play a role in dendrite and canaliculi formation, while molecules such as destrin and CapG regulate the cytoskeleton (Guo 2010). About the same time as E11 is being expressed, so is DMP1. Phex and DMP1 regulate biomineralization and mineral metabolism. Clearly, Phex is expressed before DMP1, and both regulate expression of FGF23, which targets the kidney to regulate renal phosphate excretion. Sclerostin and ORP150 appear to be markers of the late or fully embedded osteocyte. Sclerostin is a well-known marker of the mature osteocyte and is a negative regulator of bone formation (Poole *et al.* 2005). ORP150 may preserve viability of this cell in a hypoxic environment (Guo 2010).

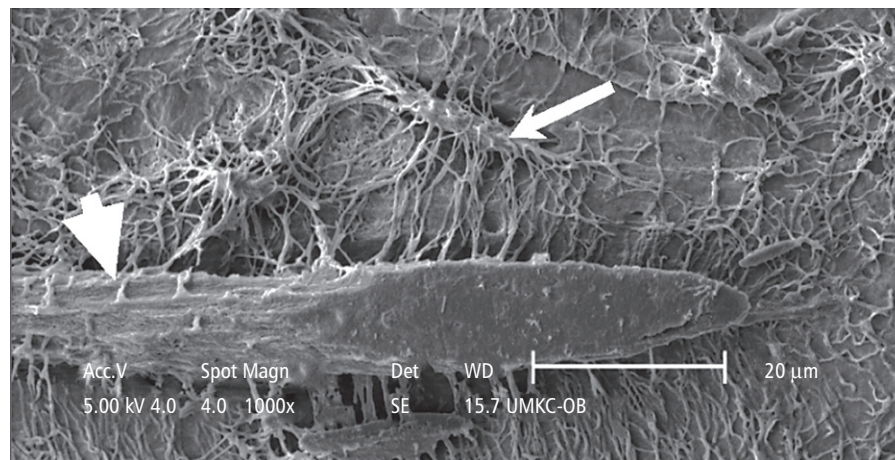


Figure 8.2 The osteocyte lacunacanalicular network is intimately connected with the bone vasculature. Murine bone embedded in resin was acid etched and then imaged by scanning electron microscopy (Feng *et al.* 2006). Note the resin-filled lacunae (arrow) and canaliculi in close association with the bone vessel (arrow head).

appear to express both RANKL and OPG (Kramer *et al.* 2010). However, one of their earliest functions was postulated to be that of mechanosensory cells of bone. For a review, see Bonewald (2006) and Klein-Nulend and Bonewald (2008). These functions will be reviewed in this chapter.

Osteocytes as mechanosensory cells

It has long been known that bone has the capacity to accommodate loading or lack of loading by modifying bone mass, and it was hypothesized that osteocytes were responsible for sensing load or lack of load. In vivo

loading has been well characterized with regard to the effects of frequency, intensity, and timing of mechanical loading on bone. It has been proposed that the response of the osteocyte to load is responsible for these changes and adaptation to load. Controversy exists regarding the best in vitro models that replicate in vivo responses. Debate continues over whether osteoblast cell lines can be used as surrogate models for osteocytes and the types of strain (hydrostatic pressure, substrate stretching, or fluid flow shear stress) the osteocyte is subjected to in vivo. However, it is generally accepted that osteocytes are subjected to fluid flow shear stress. For a review, see Klein-Nulend and Bonewald (2008).

A “bone fluid” bathes the osteocyte and its processes and travels through the osteocyte lacunocanalicular network. Blood flow is connected to and regulates the flow of the bone fluid and can be visualized with an injection of dye into the tail vein of a mouse. Penetration of the osteocyte lacunocanalicular system with dye can be observed within minutes. In addition, bone loss due to hindlimb unloading is restored with restored blood flow. Little is known about this bone fluid except that it appears that molecules larger than 70 kDa cannot travel through the osteocyte lacunocanalicular system, indicating that a sieve or molecular “cutoff” must exist between blood and the bone fluid.

At present, it is generally accepted that the osteocyte is subjected to fluid flow shear stress from the bone fluid, although type (pulsatile or oscillatory) and magnitude are still unclear. In response to bone fluid flow, osteocytes may sense shear stress through the cell body, their dendritic processes, bending of cilia, or a combination therein (Bonewald 2006). The dendritic processes may sense flow through their glycocalyx, and the cell body may sense flow through a different mechanism (Burra *et al.* 2010). The cilium, a single flagellar-like structure found on every cell, may also be involved in sensing shear stress. The Polycystin complex that clearly has a mechanosensory function in the kidney may also have a similar function in bone cells. Mice with impaired polycystin 1 function develop osteopenia due to impaired osteoblast-mediated bone formation (Xiao *et al.* 2006). However, it is not known what different signaling pathways are activated in response to shear stress of the dendrites, osteocyte cell body, and/or cilia. Depending on the types of signaling pathways activated, the osteocyte could send distinct signaling molecules with different targets.

Three small molecules rapidly released by osteocytes in response to shear stress include nitric oxide (NO), ATP, and prostaglandin. Deleting any one of these will inhibit the bone anabolic response to loading. NO inhibits bone resorption, promotes bone formation, and

appears to be released early after stress at about the same time as PGE₂ is released from osteocytes. ATP and intracellular calcium are released from osteocytes in response to extracellular calcium or mechanical stimulation. Blocking the P2X7 nucleotide receptor, an ATP-gated ion channel suppresses prostaglandin release, whereas agonists enhance release in bone cells, suggesting that the P2X7 receptor is necessary for the release of prostaglandin in response to mechanical loading. In vivo prostaglandin induces new bone formation, and indomethacin blocks the effects of anabolic loading. Prostaglandin appears to be released through hemichannels, unopposed halves of gap junction channels, in response to shear stress, which are opened via perturbation of integrin (Burra *et al.* 2010). Hemichannels may also function as transducers of the anti-apoptotic effects of bisphosphonates (Plotkin & Bellido 2001). Therefore, hemichannels in osteocytes have dual functions: release of signaling factors and protection of cell viability. For a review, see Bonewald (2007a).

The early rapid release of prostaglandin, NO, and ATP triggers activity such as glucose-6-phosphate dehydrogenase (Skerry *et al.* 1989) in a number of genes, and E11/gp38, a marker for the early osteocyte, was shown to be upregulated in response to mechanical loading (Zhang *et al.* 2006), as are Phex, MEPE, and DMP1, all of which are regulators of phosphate homeostasis (Yang *et al.* 2005; Gluhak-Heinrich *et al.* 2007). Efforts are being made to correlate the magnitude of strain with osteocyte gene expression. Sost/sclerostin, a marker for the late osteocyte and an inhibitor of osteoblast function, is downregulated by anabolic mechanical loading and increased in response to hindlimb unloading (Robling *et al.* 2008). Regulation of genes involved in osteocyte function by mechanical loading or unloading suggests that mechanical strain is not only important for skeletal health but also important for the health of other organs.

The role of the Wnt/ β -catenin pathway in osteocyte function

As outlined above, the small molecules ATP, NO, and prostaglandin are potent signaling molecules that initiate the cascade of events that lead to anabolic bone formation. What has been less clear are the signaling pathways activated by these small molecules and other late soluble signaling factors that may be released by osteocytes. A key signaling pathway activated in response to mechanical loading is the Wnt/ β -catenin pathway. This pathway is important for osteoblast differentiation, proliferation, and matrix production, whereas in osteocytes, this pathway plays a role in transmitting signals of mechanical loading to cells on the bone surface

(Bonewald & Johnson 2008). Recently it was shown that deletion of β -catenin in osteocytes results in a porous, fragile bone with a “moth-eaten” appearance (Kramer *et al.* 2010). Therefore, β -catenin is essential for the maintenance of normal bone function.

Negative regulators of the Wnt/ β -catenin pathway include Dkk1, which is expressed in all cells, and sclerostin/Sost, which is exclusively expressed in osteocytes in the adult skeleton (Poole *et al.* 2005). (The gene is *Sost*, and the protein is called sclerostin). Sclerostin is expressed in other cell types during embryonic development, but in the adult skeleton, it is expressed in mature, not early, osteocytes. Sclerostin inhibits Wnt/ β -catenin signaling by binding to Lrp5/6 and preventing the binding of Wnt. It was shown that mice lacking Lrp5 did not fully respond to anabolic loading (Sawakami *et al.* 2004).

Mutations or deletions in *Sost* and *Dkk-1* in humans and/or mice have been shown to result in increased bone mass. Mechanical loading has been shown to reduce sclerostin levels in bone, whereas hindlimb unloading has been shown to increase sclerostin expression. For a review, see Bonewald and Johnson (2008). Downregulation of Dkk1 and Sost may create a permissive environment in which Wnt proteins already present can activate this pathway. Animal studies using antibodies to sclerostin have also shown an increase in bone mass, and clinical trials have shown positive effects on anabolic bone markers suggesting that targeting of these negative regulators of the Wnt/ β -catenin signaling pathway might be useful as anabolic treatments for diseases such as osteoporosis. However, several recent studies suggest that this antibody may have greater significance and impact in accelerating the healing of bone fractures, generating new bone, and other orthopedic applications (Paszty *et al.* 2010).

It was proposed that prostaglandin, as a first response to shear stress, activates the Wnt/ β -catenin pathway in osteocytes (Bonewald & Johnson 2008) as gene transcription would be too slow for this early response. β -catenin signaling could occur independent of Wnt production and binding. It was proposed that the prostaglandin receptor EP2 activated G proteins and activated PI3-kinase, which in turn activated Akt, phosphorylating GSK-3 β , thereby inhibiting phosphorylation of β -catenin. At the same time, the G_{α} subunit bound to Axin to induce dissociation of the β -catenin degradation complex.

To test this hypothesis, *in vitro* studies were performed using MLO-Y4 osteocyte-like cells. Mechanical loading of the MLO-Y4 cells by fluid flow shear stress can protect against dexamethasone-induced apoptosis. Either prostaglandin, PGE₂, or fluid flow shear stress treatment of MLO-Y4 osteocytes resulted in increased phosphoryla-

tion of GSK-3 β , β -catenin nuclear translocation, and changes in the expression of β -catenin target genes (Kitase *et al.* 2010). The mechanism of this protective effect of mechanical loading appears to be partially mediated through PGE₂ crosstalk with the β -catenin signaling. Therefore, the Wnt/ β -catenin signaling pathway plays a role not only in bone response to loading but also in osteocyte apoptosis. Apoptosis will be discussed in this chapter.

Other pathways such as the estrogen signaling pathway and the parathyroid hormone (PTH) receptor pathway are activated in response to mechanical loading and may also crosstalk with the Wnt/ β -catenin signaling pathway. Zaman and coworkers suggest that the estrogen receptor alpha isoform (ER- α) may play a role in shuttling β -catenin into the nucleus in response to mechanical strain in osteoblasts (Zaman *et al.* 2006). This may, in part, explain how estrogen regulates bone mass through a functional intersection through ER- α with Wnt/ β -catenin signaling. Mice with an osteocyte-targeted deletion of the PTH/PTHrP receptor type 1 (PTHR1) fail to show a bone anabolic response to intermittent PTH treatment. As the PTHR1 is a G-protein coupled receptor, experiments deleting the G_{α} in osteocytes resulted in osteopenia and increased osteocyte density. Mice expressing a constitutively active PTHR1 targeted to osteocytes have increased bone cortical thickness due to increased bone formation and intracortical remodeling. Crossing these mice with mice null for the Wnt co-receptor, low-density lipoprotein-5 (Lrp5) rescued the bone formation phenotype, suggesting interplay between these pathways. Interestingly, the abnormalities in remodeling were not rescued, suggesting that although PTHR1 activation in osteocytes has effects on both bone formation and bone resorption, they are controlled by distinct mechanisms. PTH decreases Sost expression and FGF23 expression in osteocytes, demonstrating the interplay between these pathways in the control of phosphate homeostasis (Dallas & Bonewald 2010).

Osteocytes as orchestrators of bone remodeling

Not only can osteocytes regulate osteoblasts through the production of factors such as the Wnts, Dkk and sclerostin, but also osteocytes can regulate osteoclast activity through regulation of RANKL and OPG. Avian osteocytes have been shown to support osteoclast formation and activation in the absence of any osteotropic factors, as does the osteocyte cell line, MLO-Y4. RANK ligand expression on dendritic processes of the MLO-Y4 cells appeared to be responsible. Experiments using this cell line support the hypothesis that osteocytes are orchestra-

tors of bone remodeling. Not only do they send signals that regulate osteoclasts, but also conditioned media from the MLO-Y4 cells support osteoblast differentiation and, surprisingly, mesenchymal stem cell differentiation. For a review, see Bonewald and Johnson (2008).

Osteocytes appear to use different mechanisms to recruit and activate osteoclasts. Viable, necrotic, and apoptotic osteocytes can perform this function. As both isolated primary osteocytes and MLO-Y4 cells can support osteoclast formation and activation, this suggests that viable cells can be induced to send signals to osteoclast precursors. There are different forms of “dying” such as apoptosis and necrosis, and osteocytes in these states may send different signals. Targeted ablation of osteocytes through necrosis was performed on mice using the 10kb DMP1 promoter to drive the diphtheria toxin receptor expression in osteocytes (Tatsumi *et al.* 2007). Injection of a single dose of diphtheria toxin eliminated approximately 70% of osteocytes in cortical bone and generated osteoclast activation in these mice. Another model of defective osteocytes was the deletion of β -catenin in osteocytes (Kramer *et al.* 2010). Clearly β -catenin is necessary for normal osteocyte function, and deletion results in an increase in osteoclast activity and the generation of a porous bone phenotype. Lastly, osteocyte apoptosis may occur at sites of microdamage and be responsible for sending signals for removal of damaged bone. Pro-apoptotic molecules are elevated in osteocytes immediately at the microcrack locus, whereas anti-apoptotic molecules are expressed 1–2 mm from the microcrack (Verborgt *et al.* 2002). This shows that some osteocytes have protective mechanisms against apoptosis. Apoptotic osteocytes release apoptotic bodies expressing RANKL to recruit osteoclasts (Kogianni *et al.* 2008). Osteocyte cell death and cell survival are reviewed in the “Osteocyte viability and cell death” section of this chapter.

Osteocyte viability and cell death

Osteocyte viability clearly plays a significant role in the maintenance of bone homeostasis and integrity. However, whereas blocking osteocyte apoptosis may improve diseases such as bone loss due to aging or due to glucocorticoid therapy, osteocyte apoptosis may be essential for damage repair and normal skeletal replacement. Any agents that block this process may delay or worsen conditions in which repair is required. Therefore, it is important to understand and be able to identify the different states of osteocyte viability from health to compromised stressed cells, to apoptotic cells, and to necrotic cells.

Necrosis is cell death by injury where the cell is ruptured and releases its contents initiating an inflamma-

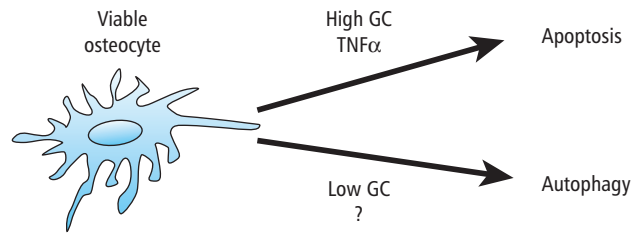


Figure 8.3 Osteocyte viability. The osteocyte can undergo apoptosis in response to a number of conditions such as unloading, immobilization, or hypoxia; cytokines such as $\text{TNF}\alpha$ and IL-1; estrogen withdrawal as in menopause; and glucocorticoid treatment. Recently it has been shown that osteocytes can also enter the state of autophagy under stressful conditions such as glucocorticoid treatment (Xia *et al.* 2010). Low-dose glucocorticoids may induce autophagy, whereas high doses may induce apoptosis (Yao, 2011).

tory event. Apoptosis is programmed cell death, an organized process where the cell content is not released and, therefore, inflammation is not initiated. A third state, autophagy, has recently been identified in osteocytes; the cell is stressed or compromised and attempts to stay alive for as long as possible before undergoing apoptosis (Figure 8.3). These states and their consequences are discussed in this chapter.

Osteocyte cell death can occur in association with pathological conditions such as osteoporosis and osteoarthritis, which lead to increased skeletal fragility. Oxygen deprivation that occurs during immobilization has been shown to promote osteocyte apoptosis as does glucocorticoid treatment and withdrawal of estrogen. $\text{TNF}\alpha$ and interleukin-1 (IL-1) have been reported to increase with estrogen deficiency and also to induce osteocyte apoptosis. For a review, see Bonewald (2007a). It may be that each of these conditions induces a percentage of osteocytes to undergo apoptosis. However, a certain number of cells may also undergo autophagy depending on the severity of the condition (Yao 2011). At any given time during a pathological process, necrosis, apoptosis, and autophagy could be occurring simultaneously resulting in different osteocyte function and signaling.

Because osteocyte cell death, especially apoptosis, has been associated with negative effects on bone, investigations into apoptosis inhibitors have taken a lead. Inhibitors of osteocyte cell death include estrogen and selective estrogen receptor modulators, bisphosphonates, calcitonin, CD40 ligand, calbindin-D28k, and monocyte chemotactic proteins (MCP) 1 and 3. For a review, see Bonewald (2007a). Recently, mechanical loading in the form of fluid flow shear stress that mimics bone fluid flow in the osteocyte lacunocanalicular network has been shown to block glucocorticoid-induced apoptosis

(Kitase *et al.* 2010). This was shown to be mediated through the release of prostaglandin, which activated the Wnt/ β -catenin pathway. Alternatively, it has been postulated that the reason for the opposite effects of mechanical loading and glucocorticoid on apoptosis of osteocytes is due to their opposing actions on kinases of the focal adhesion family–focal adhesion kinase (FAK) and the proline-rich tyrosine kinase 2. It was also proposed that extracellular matrix interactions with integrins leading to focal adhesion kinase signaling is linked with the Wnt/ β -catenin pathway (Bellido 2010). Therefore, whether mechanical loading is applied through fluid flow shear stress or through substrate stretching, the Wnt/ β -catenin pathway appears to be involved.

In addition to undergoing programmed cell death, osteocytes can undergo a process of self-preservation called *autophagy*, especially in response to glucocorticoid (Xia *et al.* 2010). Autophagy is a lysosomal degradation process necessary for recycling cellular products. During autophagy, parts of the cytoplasm and intracellular organelles are incased in autophagic vacuoles that fuse with lysosomes to be degraded. Autophagy can preserve viability until the cell can return to normal or non-stressed conditions or, alternatively, can be a self-destructive process that leads to cell death if the stress continues. Therefore, osteocytes undergoing autophagy can return to normal, undergo apoptosis, or perhaps even undergo necrosis.

Osteocyte cell death may be responsible for some forms of osteonecrosis. Osteonecrosis is “dead” bone containing empty osteocyte lacunae that do not remodel. Dead bone that does not remodel can be present in the skeleton for years. Early proposed mechanisms responsible for osteonecrosis include (1) the *mechanical theory*, where osteoporosis and the accumulation of unhealed trabecular microcracks result in fatigue fractures; (2) the *vascular theory*, where ischemia is caused by microscopic fat emboli; and (3) a newer theory, *osteocyte apoptosis*, where agents induce osteocyte cell death resulting in dead bone that does not remodel (Bonewald 2007a). As reviewed above, viable osteocytes are necessary to send signals of (re)modeling. Whether osteocyte apoptosis leading to empty lacunae in osteonecrosis of the jaw due to intravenous bisphosphonates is the cause or the result of the disease is still debated (Allen & Burr 2009).

Medical, dental, and health implications

Osteocyte health, viability, and capacity to regulate its own death most likely play highly significant roles in the maintenance and integrity of bone. Bone loss in osteoporosis and lack of bone remodeling with osteonecrosis may be due to pathological osteocyte cell death. It will

be important to develop therapeutics that maintain both osteocyte viability and the normal physiological osteocyte cell death that leads to normal bone repair.

The connectivity and structure of the osteocyte lacunocanalicular system may play a role in bone disease. Variability in complexity and number of dendrites and canaliculi could have a dramatic effect on osteocyte function and viability, the mechanical properties of bone, and the capability of bone to respond to mechanical loading. In osteoporotic bone, there is disorientation of the canaliculi as well as a marked decrease in connectivity that increases in severity. In osteoarthritic bone, a decrease in connectivity is observed, but orientation is intact. In osteomalacic bone, the osteocytes appear viable with high connectivity, but the processes are distorted and the network chaotic (Knothe Tate *et al.* 2004). A compromised lacunocanalicular system may not perform optimally to the anabolic loading of bone.

Understanding how the osteocyte lacunocanalicular system operates as an endocrine organ regulating phosphate metabolism should lead to insight into diseases of hyper- and hypophosphatemia. Any condition that compromises the osteocyte’s capacity to maintain the normal equilibrium of bone remodeling would lead to excess bone formation or excess bone resorption. Obviously compromised alveolar, maxillary, and mandibular bone function affects maintenance of dental function and oral health. At this time, it is not known if osteocytes in these bone tissues have similar or different properties to those in unloaded bone such as the calvaria or loaded bone such as the vertebrae and long bones.

Conclusions, future directions, and perspectives

In conclusion, though little was known about the biology and function of osteocytes for decades compared to osteoblasts and osteoclasts, knowledge in this field is rapidly expanding. Predicted functions such as being mechanosensory cells and the capacity of osteocytes to remodel their perilacunar matrix are being validated, and the responsible molecular mechanisms and signaling pathways identified. Novel functions such as regulators of osteoclasts and osteoblasts have provided new insights into the function of osteocytes as orchestrators of bone remodeling. The observation that these cells can produce factors that target other organs and that the osteocyte network functions, therefore, as a unique endocrine gland is surprising. The potential therapeutic use of neutralizing antibody to sclerostin, a marker of late osteocytes, has emphasized the importance of understanding this bone cell type. This antibody may prove useful in orthodontic applications. It will be important

to continue to study and follow discoveries made regarding this bone cell.

Summary

Osteocytes are a subpopulation of differentiated osteoblasts that have become embedded in the mineralized bone matrix. The biology and function of osteocytes were unclear and highly debated for decades. Some investigators proposed their major function to be in mechanosensation by transmitting load or absence of load to cells on the bone surface to regulate bone mass. At the same time, diametrically opposed researchers described osteocytes as passive cells simply acting as placeholders in bone. This controversy continued until new technologies were developed and used to determine that osteocytes possess important biological functions. In the last decade, osteocytes have been shown to be multifunctional cells acting as regulators of bone remodeling and regulators of systemic mineral homeostasis, among other functions. Maintenance of osteocyte health is necessary to maintain a healthy adult skeleton.

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Clinical correlate: Van Buchem disease

H.-J. Prins, A.L.J.J. Bronckers, and J. Klein-Nulend

Van Buchem disease (VBD) was first described in 1955 and was originally named hyperostosis corticalis generalisata. It is an extremely rare autosomal recessive sclerosing bone dysplasia (Vanhoenacker *et al.* 2000), presenting as an increase in cortical bone thickness and density affecting the skull, mandible, and long bones. Therefore, VBD has been classified as craniotubular hyperostosis (Beighton *et al.* 2007). The prevalence of VBD is very low, and in the 1990s fewer than 30 patients were described, predominantly in the Dutch population (reported by Van Buchem). More specifically, 13 VBD patients were detected in a highly inbred Dutch family that has a common ancestor and lives in a small ethnic isolated village (Van Hul *et al.* 1998). Later, only a minimal number of additional cases, with only a few cases in other parts of the world, have been reported. The condition is usually detected in childhood. Since increased bone formation is more prominent in the elderly, it appeared that VBD progressed with aging but with minimal physical impairment.

Genetic background

Mutations in the region of the *SOST* gene on chromosome 17q12-21 have been identified as giving rise to two similar diseases, VBD and the much more severe sclerosteosis. Sclerosteosis patients have mutations in the *SOST* coding region resulting in the absence of the functional gene product, sclerostin (Figure 9.1A). In contrast, no mutations were detected in the *SOST* coding region of patients with VBD (Brunkow *et al.* 2001), but a 52 kb deletion approximately 35 kb downstream of the *SOST* gene (Figure 9.1B; Van Hul *et al.* 1998; Balemans *et al.* 2002). Analysis of the sequences flanking the deletion

breakpoints showed the presence of Alu-repeats (repetitive mobile elements in the human genome), suggesting an Alu-mediated, unequal homologous recombination event as the mechanism causing the deletion. As no coding sequences could be identified within the deleted region, it was suggested that loss of specific regulatory elements may downregulate the *SOST* expression in VBD patients (Balemans *et al.* 2002; Loots *et al.* 2005).

Sclerostin: characteristics and expression

The *SOST* gene consists of two exons encoding the 213-amino acid secreted sclerostin glycoprotein. It contains a cysteine knot motif involved in dimerization plus receptor binding and a signaling peptide for secretion. *SOST* mRNA, particularly during embryogenesis, is expressed in many tissues, while postnatal sclerostin protein expression is observed only in osteocytes, mineralized hypertrophic chondrocytes, and cementocytes. As expected for an osteocyte-derived secreted protein, high levels of sclerostin are detected in the lacunar-canalicular network. In VBD patients, none of these cell types express sclerostin (Winkler *et al.* 2003; van Bezooijen *et al.* 2009).

Sclerostin belongs to the evolutionary-conserved DAN (differential screening-selected gene aberrative in neuroblastoma) family of glycoproteins, sharing the ability to affect the activity of several growth factors, including bone morphogenetic protein (BMP) and Wnts.

Sclerostin as an inhibitor of bone formation

Among bone cells, sclerostin is found almost exclusively in the osteocytes. Sclerostin binds to the two β -propeller

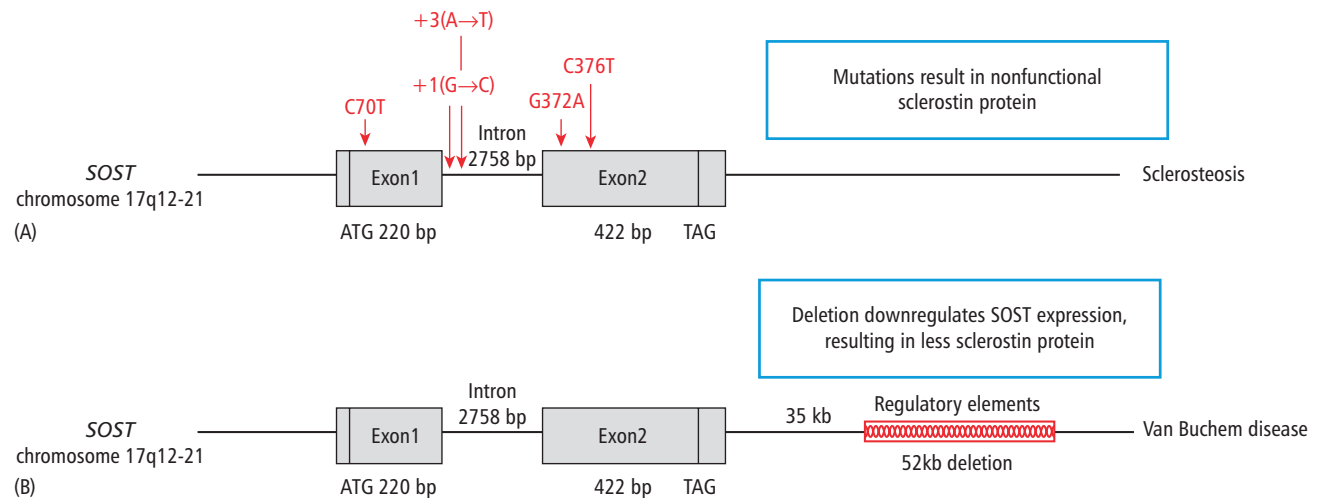


Figure 9.1 Schematic illustration of the *SOST* gene that is located at chromosome 17q12-21 and consists of two exons (220 and 422 bp, respectively). (A) In patients with sclerosteosis, three mutations (C70T, G372A, and C376T) have been found in the two exons, leading to a premature end of translation, resulting in a nonfunctional gene product, sclerostin, and two base substitutions in the intron (+1 G→C and +3 A→T), all of which resulted in improper splicing of the coded message. (B) In patients with Van Buchem disease, a 52 kb deletion is found approximately 35 kb downstream of the *SOST* gene. The deletion region probably contains specific regulatory elements that may downregulate *SOST* expression in Van Buchem disease patients.

domains of the Wnt co-receptors LRP5 and LRP6, thereby antagonizing Wnt/ β -catenin signaling by inhibiting β -catenin nuclear translocation and transcription of Wnt target genes (Nusse 2005; Semenov *et al.* 2005). The antagonistic effects of sclerostin on LRP5/6 receptor signaling provide a potential mechanism for the osteocytes to control mechanotransduction, by adjusting their sclerostin (Wnt inhibitory) signal output to modulate Wnt signaling in the effector cell population. Besides its inhibitory effect on the Wnt-signaling pathway, sclerostin can also inhibit BMP/SMAD signaling. Sclerostin interacts intracellularly with both mature domain and pro-domain of BMP-7, resulting in proteasomal degradation and thereby blocking its secretion in osteocytes (Krause *et al.* 2010). In addition, sclerostin stimulates apoptosis of osteoblastic cells, providing another mechanism by which sclerostin may inhibit bone formation (Winkler *et al.* 2003; Sutherland *et al.* 2004). By keeping both Wnt/ β -catenin and BMP7/SMAD in check, sclerostin plays an important role in maintaining bone homeostasis (Figure 9.2A). Without sclerostin, the negative feedback on osteoblast activity is absent, like in VBD, which results in excessive bone formation (Figure 9.2B).

Mechanical loading regulates sclerostin expression in osteocytes

Bone adapts its mass and shape in response to increased mechanical loading or lack of loading (disuse), thereby dynamically adjusting skeletal strength throughout life. Sclerostin is expressed in mechanosensitive osteocytes,

and evidence for mechanoregulation of sclerostin expression was reported in mice and rats subjected to ulnar loading *in vivo* (Robling *et al.* 2008). Modulation of sclerostin levels appears to be a finely tuned mechanism by which osteocytes coordinate regional and local osteogenesis in response to increased mechanical stimulation (Figure 9.2A), perhaps via releasing the local inhibition of Wnt/Lrp5 signaling by sclerostin. The activation of the Wnt/ β -catenin pathway in osteocytes occurs via a concerted mechanism. Mechanical loading increases nitric oxide (NO) production as well as activates focal adhesion kinase (FAK) and the Akt signaling pathway, which results in β -catenin stabilization, followed by β -catenin translocation to the nucleus, and expression of β -catenin target genes such as CD44, connexin 43, cyclin Dd1, and c-fos (Santos *et al.* 2010). The propagation of this signal occurs after induction of Wnt production by mechanical loading, which results in reactivation of the Wnt/ β -catenin signaling pathway (Santos *et al.* 2009). The position of the osteocytes can affect their production of sclerostin. Osteocytes close to the surface, and therefore probably exposed to a more intense mechanical stimulation, are mostly sclerostin negative while osteocytes deeper in the tissue are mostly sclerostin positive. Osteocytes in the close proximity to an area of bone formation are also mostly sclerostin negative (Poole *et al.* 2005), suggesting that not only new bone formation depends on sclerostin distribution, but also bone formation during remodeling might be dependent on the local position of sclerostin-producing osteocytes.

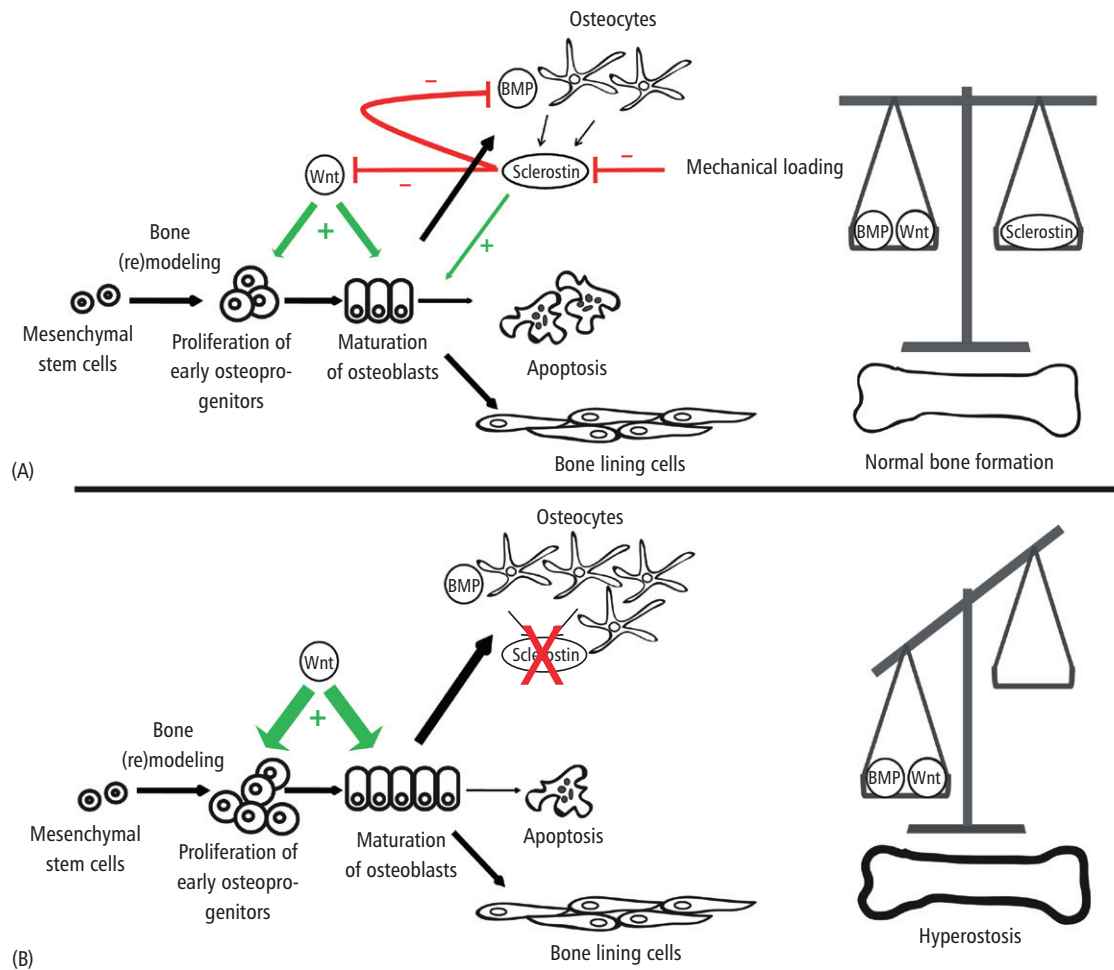


Figure 9.2 Schematic illustration of the effects of sclerostin on bone formation. (A) In a normal situation, sclerostin is produced and secreted by osteocytes, antagonizes LRP5/6-mediated Wnt signaling, and thereby inhibits the stimulatory effect of Wnts on osteoblast proliferation and maturation. Sclerostin also negatively regulates BMP7/SMAD signaling intracellularly in osteocytes that express sclerostin. BMP and Wnt pathways cooperate in stimulating bone formation, and, therefore, sclerostin plays a key role in balanced bone (re)modeling by antagonizing both BMP and Wnt signaling. The expression of sclerostin is downregulated by mechanical loading, thereby providing a mechanism by which bone formation is increased. (B) In VBD and sclerosteosis patients, sclerostin is absent. Without the inhibiting effects of sclerostin on bone formation and less stimulation of apoptosis, increased BMP- and Wnt-induced bone formation results in hyperostosis and high bone mass.

Clinical features

General

Sclerosteosis becomes evident in early childhood (i.e., mandibular prognathism and increased frontal prominence have been reported as early as five years of age). VBD, on the other hand, is diagnosed in the second decade of life as moderate thickening of the skull base, mandible, and calvaria (disappearance of diploë) (Figure 9.3A–C; Fryns & Van den Berghe 1988). A characteristic feature of both sclerosteosis and VBD is the enlargement of the jawbone that continues to expand throughout life, indicating that the defects are not developmental defects but involve the ongoing activity of osteoblasts through-

out life. In sclerosteosis patients, the changes are much more severe than in VBD patients. A comparison of the characteristic clinical features of VBD and sclerosteosis is provided in Table 9.1. Syndactyly and tall stature are not observed in patients with VBD but are commonly seen in sclerosteosis, which makes it easier to distinguish one disease from the other.

The diagnostic radiographic features of sclerosteosis and VBD are symmetrical massive thickening and enlargement of the bones, most frequently found as an enlarged mandible but also an enlarged skull (vault and base), clavicles, ribs, and pelvis, and diaphysis of long bones and tubular bones of the hands and feet (Figure 9.3D; Wergedal *et al.* 2003). The long bones show cortical

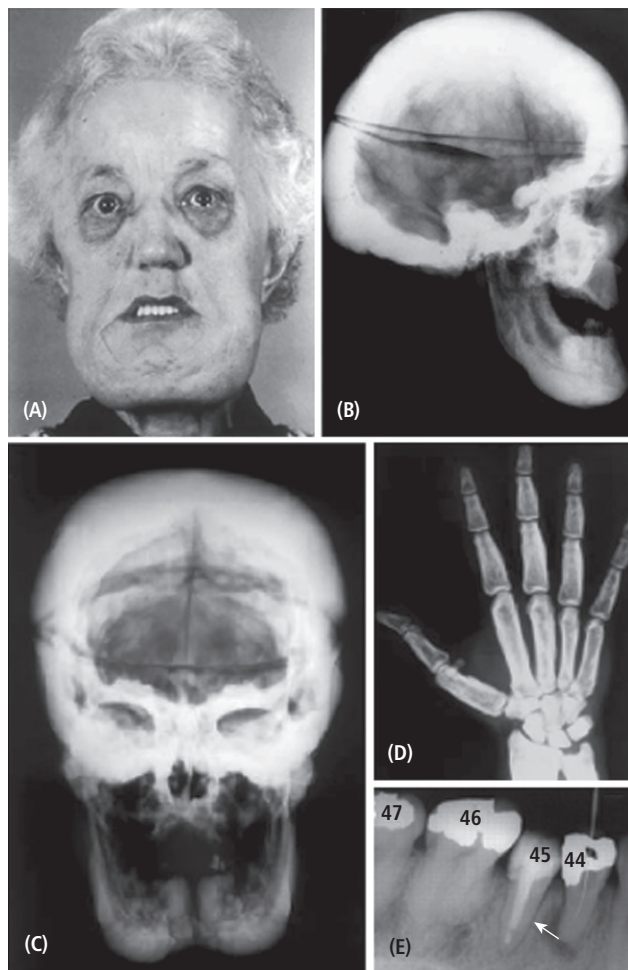


Figure 9.3 Photographs and radiographs of patients with VBD. (A) VBD patient with a protruding chin. (B–C) Anteroposterior and lateral view of a postmortem skull of a patient with VBD. Extensive sclerosis of the calvaria and skull base and enlargement of the mandible are visible. There is loss of diploë of the calvaria. (D) Radiograph of the right hand of a 52-year-old male patient, showing endosteal thickening of the diaphyseal cortices of the short tubular bones with obliteration of the medullary canal, and disturbance in modeling. (E) Radiograph of premolars (44 and 45) and molar teeth (46 and 47) of a 43-year-old female patient with VBD. The premolar (45) shows a radiolucent periodontal space not seen at the first and second molars (46 and 47). (Figure 9.3A–C is reprinted from Van Hul *et al.*, 1998, with permission from Elsevier. Figure 9.3D is reprinted from Vanhoenacker *et al.*, 2000, with permission from Springer Science+Business Media. Figure 9.3E is reprinted from Van Bezooijen *et al.*, 2009, with permission from SAGE Publications.)

thickening of the shafts resulting in a narrowing of medullary cavities. Most clinical reports on sclerosteosis and VBD indicate that the increase in bone volume occurs at the endosteal side. Dynamic labeling studies in animals injected with anti-sclerostin indicated, however, that both periosteal and endosteal bone formation increase

significantly and do not exclusively take place at the endosteal side (Ominsky *et al.* 2010).

The bone contours are disrupted due to many subperiosteal osteophytes (exostoses) resulting in a very rough bone surface. The clinical consequence of hyperostosis of the skull is narrowing of the foramina, that causes entrapment of the seventh cranial nerve, which often leads to facial palsy, and entrapment of the eighth cranial nerve, which often results in deafness, neurological pain, visual problems, and, in some cases, blindness as a consequence of optic atrophy. Annual assessment from infancy is recommended for patients with impaired hearing, as it is evidence of nerve entrapment and increased intracranial pressure.

VBD patients have increased metacarpal outer diameter, inner diameter, cortical thickness, and bone mineral density that results in increased bone strength. Fractures and haematological changes are not found in VBD patients. Laboratory values are normal, except for several biochemical indices of bone turnover such as elevated serum alkaline phosphatase levels. Serum procollagen 1 peptide, osteocalcin, and urinary type I collagen cross-linked N-telopeptide are increased in some but not all cases.

Orofacial bone and dental aspects

There is no evidence that loss of function of *SOST* directly affects the development of the dentition. Rather, defects in dentition, which are reported occasionally in patients suffering from osteosclerosis, seem to be secondary. There is one report claiming that tooth eruption is delayed (Stephen *et al.* 2001). There is no evidence that orthodontic tooth movement in sclerosteosis or VBD patients is slower than in healthy subjects as might be expected in sclerosteosis, since this disease develops during early childhood. The bone thickening progresses with age, and the bone is hard and resistant to fracture. Therefore, tooth extraction may be difficult and management by an orthodontic or craniofacial team is recommended (Beighton *et al.* 2007).

Roots of teeth of sclerosteosis patients contain massive layers of cellular cementum, which is the bone-like tissue that attaches the collagen type I fibers of the periodontal ligament to the root dentin. For a review, see Chapter 20 (“Cementum”) in this volume. Cellular cementum contains cementocytes, similar to bone that contains osteocytes, but unlike bone, cementum is not remodeled, it does not contain vessels, and the canalicular network is less well developed. Hyperostosis and hypercementosis can result in ankylosis—a bone-like tissue connecting root dentin and alveolar bone. Cementocytes from healthy mice and humans express sclerostin (Van Bezooijen *et al.* 2009; Jaeger *et al.* 2010), while those from sclerosteosis and VBD patients fail to do so or express strongly reduced levels (Van Bezooijen *et al.* 2009).

Table 9.1 A comparison of characteristic clinical features of VBD and sclerosteosis. (Adapted from Beighton *et al.*, 2007, with permission from the University of Washington, Seattle. Available at <http://www.genetests.org>.)

	Osteosclerosis	Van Buchem Disease
Chromosome <i>SOST</i> gene	17q12-21	17q12-21
OMIM	269500	239100
Genetic mutations	In <i>SOST</i> gene	52 kb deletions 35 kb downstream
Inheritance	Autosomal recessive	Autosomal recessive
Clinical testing method	Sequence analysis for <i>SOST</i> sequence variants	Targeted mutation analysis for deletion
Age of clinical presentation	Early childhood	Puberty
Prognosis	Potentially lethal	Comparatively benign
Habitus	Gigantism	Normal stature
Facies	Gross distortion	Prominent mandible
Teeth	- Irregular, with dental malocclusion - Widely spaced teeth - Excess cellular cementum	- Normal dental occlusion - No obvious excess cellular cementum
Opening mouth	Difficult, poor development of the mandible angle	
Cranial nerve palsy	Very common, entrapment of nerves	Inconsistent
Hearing problems	Yes	No
Proptosis	Yes	No
Intracranial pressure	Raised	Normal
Syndactyly of the fingers	Frequent	Absent
Distortion of tubular bones of hands and feet, cortical hyperostosis	Marked	Mild
Nail hypoplasia	Frequent	Absent
Cranial hyperostosis	Gross	Moderate
Bone resorption marker		Increased
- Urinary type I N telepeptide		
Serum markers bone formation	Elevated	Elevated
- Alkaline phosphatase		
- Type I procollagen peptide		
- Osteocalcin		

However, X-ray images from sclerosteosis and VBD patients do not show clear signs of ankylosis, although the identification of periodontal gaps is not always possible owing to the very dense radio-opacity of the overlying bone (Figure 9.3E; Van Bezooijen *et al.* 2009).

Orofacial bone versus tubular bone

The prominent skull and mandibular bone growth in sclerosteosis and VBD patients might be related by potential differences in osteoblasts at different skeletal sites, although this has received little attention so far. Osteoclasts and osteocytes from craniofacial bones differ from osteoclasts in the appendicular skeleton (long bones of extremities) regarding the expression of molecules and sensitivity for loading (Vatsa *et al.* 2008; Zenger *et al.* 2010). Calvarial bone and long bone also

differ in composition, suggesting heterogeneity between osteoblasts from both skeletal sites. Whether osteoblasts of craniofacial bone (intramembranous bone of different embryological origin) are more sensitive to loss of sclerostin or whether osteocytes from calvarial bone or jawbone produce more sclerostin than osteocytes in long bones is unknown. Differences in the magnitude of mechanical loading on long bone versus craniofacial bone may also play a role.

Therapeutic possibilities

Surgical removal of excessive bone

Surgical treatment aiming at the removal of excess bone is mostly used to treat VBD. This surgical treatment is technically difficult and sometimes dangerous (Marmary

et al. 1989; du Plessis 1993), and it might include surgical decompression of entrapped cranial nerves, craniectomy for increased intracranial pressure, middle ear surgery for conductive hearing loss, and/or surgical reduction of mandibular overgrowth. These treatments aim to relieve symptoms, but they do not include a systemic approach to counteract the underlying hyperostosis. Testing of relatives at risk is recommended and includes clinical appraisal, lateral skull radiography if indicated, and targeted mutation analysis for the deletion.

Glucocorticoids

Glucocorticoids might be an attractive alternative to the high-risk surgical procedures that are used as treatment for patients with progressive sclerosing bone disorders (Van Lierop *et al.* 2010). Glucocorticoids decrease bone formation by inhibiting proliferation and differentiation of osteoblasts and increasing their rate of apoptosis (Weinstein *et al.* 1998). Van Lierop and colleagues (2010) suggest that sclerostin is involved not only in bone formation, but also in the regulation of bone resorption. The exact mechanism by which sclerostin may be involved in the regulation of bone resorption is yet to be explored. These observations suggest that glucocorticoids could be used as an additional, systemic therapy in patients with increased risk of neurological complications due to bone overgrowth like VBD.

Sclerostin antibody as a bone-forming agent

The inhibition of bone formation by sclerostin seen in studies of sclerosteosis and VBD patients gained interest from researchers in the field of osteoporosis who were searching for new bone-forming agents. Inhibition of sclerostin by injection of antibodies has already been shown to increase bone formation, bone mass, and bone strength in animal models, including primates (Li *et al.* 2010; Ominsky *et al.* 2010). A first phase I clinical study demonstrated that a single injection of a monoclonal antibody against sclerostin markedly increases bone formation markers and bone density, decreases bone resorption, and is well tolerated (Padhi *et al.* 2010). These observations suggest that pharmacologic inhibition of sclerostin may represent a promising anabolic therapy for disorders related to low bone mass.

Summary

Van Buchem disease (VBD) is an extremely rare hereditary disorder characterized by hyperostosis of the bones of the skull, mandible, clavicles, ribs, and long bones. Loss of sclerostin expression in osteocytes leads to abnormal bone formation in patients with this disease. In this chapter, the genetic background of the disease that involves the *SOST* gene, its product sclerostin, the

clinical features caused by *SOST* mutations, and the therapeutic possibilities are discussed.

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Stem cell biology in the craniofacial apparatus

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Stem cells have been used in diverse medical fields for the regeneration and/or repair of defective tissues and organs such as bone, ligaments, or heart. Most of these therapeutic approaches are based on knowledge gained from studies of embryonic development. In fact, the goal of regenerative medicine is to mimic the mechanisms and processes that occur in nature during the initiation and morphogenesis of a specific organ. Hence, stem cells offer an impressive potential to be useful in the treatment of diseases and malformations affecting the craniofacial region. One of the key characteristics of craniofacial development is the formation of cranial neural crest cells (CNCCs). Neural crest cells (NCCs) are a migratory cell population that is unique to vertebrate embryos and gives rise to a wide variety of differentiated cell types. The induction, migration, proliferation, survival, and ultimate fate of CNCCs play a crucial role in regulating normal development of the craniofacial region, as perturbation of any of these processes may lead to an array of pathologies (Dixon *et al.* 2006; Etchevers *et al.* 2006).

Early development of CNCCs

Induction and delamination

The neural crest is induced at the dorsolateral border of the neural folds between the surface ectoderm and the neural plate via molecular interactions across this interface (Selleck & Bronner-Fraser 1995; Sauka-Spengler & Bronner-Fraser 2008). At least four key signaling pathways play a critical role in this process as they intersect at the neural plate border (Basch & Bronner-Fraser 2006). A gradient of bone morphogenetic protein (BMP)

signaling within the neural plate has been proposed as a requirement for NCC induction in various vertebrate species (Marchant *et al.* 1998; Wawersik *et al.* 2005). In addition, Wnt signaling from the ectoderm and fibroblast growth factor (FGF) signaling from the underlying mesoderm also possess the ability to induce NCC formation (Monsoro-Burq *et al.* 2003; Lewis *et al.* 2004). Notch signaling is required upstream of BMP expression to promote NCC formation (Endo *et al.* 2002).

Simultaneously with their induction, NCCs undergo epithelial-to-mesenchymal transformation (EMT), which results in their delamination and subsequent migration from the neural tube. At this stage, NCCs become progressively specified and express various markers. Among them, Snail1, Snail2, Sox8, Sox9, Sox10, FoxD3, AP-2, Twist, c-Myc, and Id family members are the most noteworthy (Duband 2006; Sakai *et al.* 2006; Sauka-Spengler & Bronner-Fraser 2006, 2008). Some of these transcription factors are involved in the regulation of events leading to EMT, including changes in cell adhesive properties, motility, and cell cycle progression. For instance, Snail2 promotes EMT through repression of E-Cadherin, which occurs concomitantly with other changes in the expression of diverse cell adhesion molecules (Nakagawa & Takeichi 1998; Nieto 2002; Taneyhill *et al.* 2007). Accordingly, it has been proposed that a controlled balance of cadherin expression by NCCs determines their status as premigratory or migratory populations (Nakagawa & Takeichi 1998; Taneyhill 2008).

Adhesion and other events related to EMT are also controlled by extracellular signals, which include extracellular matrix (ECM) proteins as well as secreted transforming growth factor β (TGF β) and FGF ligands.

Activation of these signaling pathways triggers the expression of transcription factors and other genes. For instance, BMP signaling induces the expression of Snail, but it also induces that of several cadherins and RhoB in a specific temporal sequence (Liu & Jessell 1998). Moreover, the transition from epithelial to mesenchymal phenotype depends upon cells shifting from G1 to S-phase, which is dependent on BMP signaling (Burstyn-Cohen *et al.* 2004). Another necessary step to complete a full EMT and allow the migration of NCCs is the breakdown of the basement membrane that surrounds the neural tube (Acloque *et al.* 2009), which appears to be mediated by matrix metalloproteinases (Parsons *et al.* 1997; Robbins *et al.* 1999).

Regionalization and migration

Once NCCs are induced and delaminated, they are able to migrate to specific destinations. The neural crest is subdivided into four distinct axial populations—cranial, cardiac, vagal, and trunk—each of which contributes to particular cell and tissue types. CNCCs can be further subdivided into forebrain-, midbrain- and hindbrain-derived populations. The segmented nature of the hindbrain into rhombomeres (r) provides an additional structural framework for migration. CNCCs have been shown to migrate as streams, three of which can be identified in all vertebrate embryos: trigeminal, hyoid, and postotic. The trigeminal crest arises from the midbrain and r1 and r2 of the hindbrain and forms neurons within the trigeminal ganglion and the components of the frontonasal prominence and the first branchial arch (BA) (Lumsden *et al.* 1991; Schilling & Kimmel 1994). The second crest stream, the hyoid, arises primarily from r4 and forms neurons of the proximal facial ganglion as well as the constituents of the second BA (Lumsden *et al.* 1991; Schilling & Kimmel 1994). Finally, the postotic crest is generated by r6 and r7 and forms the neurons of the proximal and jugular ganglia and the skeletal components of the posterior BAs (Lumsden *et al.* 1991; Schilling & Kimmel 1994).

After regionalization of the neural tube, the segmental streaming of CNCC migration into the BAs is controlled locally by a combination of intrinsic factors and paraxial exclusion zones in the ectoderm and mesoderm, which restrict the migration of CNCCs through the territory adjacent to the odd-numbered rhombomeres (r3 and r5) (Farlie *et al.* 1999; Golding *et al.* 2002; Trainor *et al.* 2002; Kulesa & Gammill 2010). Many guidance molecules within the local environment play a role in early CNCC migration, shaping and maintaining the early features of the CNCC streams. These include ErbB4 (Golding *et al.* 2000, 2004), Eph–ephrin interactions (Davy & Soriano 2005; Mellott & Burke 2008), chemokines (Killian

et al. 2009), and neuropilin–semaphoring interactions (Gammill *et al.* 2007; Schwarz *et al.* 2008). The population of target destinations by CNCCs and their proper assembly into differentiated structures are also highly regulated events that involve multiple guidance cues, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). These factors act as strong chemoattractive cues for CNCCs (Eberhart *et al.* 2008; McLennan *et al.* 2010). Twist, Tbx1, and FGFs are also required for the proper segregation of CNCC streams populating the first and second BAs (Soo *et al.* 2002; Moraes *et al.* 2005; Trokovic *et al.* 2005). The action of guidance molecules and their interaction at different phases of the CNCC migration program suggest a strategy of chemoattraction and local inhibition (Gammill *et al.* 2007; McLennan & Kulesa 2007; McLennan *et al.* 2010).

Postmigratory CNCCs in the development of diverse craniofacial structures

The analysis of Wnt1-Cre;R26R transgenic embryos has provided valuable information regarding the contribution of postmigratory CNCCs to the head in mammals (Chai *et al.* 2000; Chai & Maxson 2006). Upon arrival of CNCCs at their destination in the ventral region of the embryo, their proliferative activity produces the discrete swellings that demarcate the first and second BAs as well as the frontonasal prominence. A distal-less homeobox (Dlx) code provides CNCCs with patterning information and intra-arch polarity along the dorsoventral (DV)/proximodistal axis. In each BA, Dlx1/2, Dlx5/6, and Dlx3/4 transcripts overlap distally but display offset proximal expression limits. In the first BA, Dlx1 and Dlx2 are expressed in both the maxillary and mandibular processes. Dlx5 and Dlx6 are expressed only in the mandibular process (Depew *et al.* 2005). Dlx3 and Dlx4 expression domains are further restricted to the distal-most end of the mandibular process (Depew *et al.* 1999, 2002; Jeong *et al.* 2008). Thus, the partitioning of the first BA is mainly achieved with two Dlx combinations: Dlx1/2 for the maxillary process and Dlx1/2/5/6 for the mandibular process (Depew *et al.* 2005; Jeong *et al.* 2008; Minoux *et al.* 2009; Minoux & Rijli 2010). Once specified, maxillary and mandibular prominences are established through local migration and regionalized proliferation of CNCC. In this way, the primitive face and mouth are formed. The frontonasal prominence contributes significantly to the formation of the nose and the upper lip. The maxillary process gives rise to part of the upper lip, the maxillary bone, and the secondary palate, whereas the mandibular prominence forms the mandible. A great number of studies in embryology have focused on palatogenesis because defects in this process

lead to cleft palate, one of the most common birth defects in humans. CNCCs are critical for palate development as they contribute about 95% of the total number of mesenchymal palatal cells, whereas the remaining 5% corresponds to mesoderm-derived cells (Chai *et al.* 2000, n.d.). An interesting example of the influence of CNCCs in palate formation is observed in *Tgfr2^{fl/fl}*; *Wnt1-Cre* mutants. The loss of the TGF β signaling pathway in neural crest derivatives produces a severe cleft palate caused by reduction in proliferation of CNCC–mesenchyme-derived cells, which involves complex intracellular signaling modifications (Ito *et al.* 2003; Iwata *et al.* n.d.).

To pattern craniofacial structures, CNCCs interact actively with other tissues including the oral and non-oral ectoderm (Lumsden & Krumlauf 1996; Brugmann *et al.* 2006). By providing critical feedback to these tissues, CNCCs supply species-specific patterning information during craniofacial development, highlighting the importance of tissue–tissue interaction in regulating organogenesis (Schneider & Helms 2003; Helms *et al.* 2005; Brugmann *et al.* 2006). In addition to the developing palate, tooth development is another excellent example of the consistent shift of instructive signals between CNCC-derived mesenchyme and oral epithelium. The initial inductive signal for tooth formation appears to reside in the oral epithelium at the lamina stage. Later this odontogenic potential shifts into the CNCC-derived mesenchyme, which is able to induce tooth formation even when combined with a nondental epithelium. Subsequently, odontogenesis is directed by a specific group of signaling epithelial cells, known as the *enamel knot* (Tucker *et al.* 1998; Tucker & Sharpe 2004; Thesleff 2006). CNCCs appear to act at two different points in the development of craniofacial structures, particularly in odontogenesis. First, CNCCs as a group contribute to the formation of condensed dental mesenchyme at the initial budding stage of tooth development and provide patterning information for the progression of tooth morphogenesis. Through the critical and continued interactions between the oral epithelium and CNCC-derived dental mesenchyme, the size, shape, and number of teeth are also determined during development (Tucker & Sharpe 2004; Chai & Maxson 2006). Second, CNCCs differentiate into specific cells and tissues: dentin-matrix producing odontoblasts and cells composing pulp, cementum, alveolar bone, and periodontal ligament of maxillary and mandibular teeth (Chai *et al.* 2000).

A number of studies have provided strong evidence to support the hypothesis that the pattern of postmigratory CNCCs *as a group* is plastic and appears to be instructed by signals not only from the ectoderm but also from the

pharyngeal endoderm, the mesoderm, and, earlier, the isthmus organizer at the midbrain–hindbrain boundary (Couly *et al.* 2002; Trainor *et al.* 2002; Le Douarin *et al.* 2004). The pharyngeal endoderm makes a limited contribution to craniofacial development, but it serves as an inducer during tissue–tissue interactions. Previous studies have shown that manipulation of the pharyngeal endoderm and FGF pathway in this tissue at early stages results in defects in facial bone and cartilage development (Couly *et al.* 2002; Ruhin *et al.* 2003; Crump *et al.* 2004; Helms *et al.* 2005). Moreover, the development of the thyroid, parathyroid, and thymus involves the interaction of the pharyngeal endoderm and its flanking CNCCs (Graham *et al.* 2005). Compromised RA signaling from the pharyngeal mesoderm affects the development of pharyngeal endoderm, which in turn causes defects in CNCC migration and the development of pharyngeal pouch-derived organs, such as the thymus and parathyroid glands (Niederreither *et al.* 2003). Additionally, during the development of the first BA, the pharyngeal endoderm is thought to pre-pattern the orofacial epithelium, which in turn provides instructive signals to pattern the CNCC-derived mesenchyme (Haworth *et al.* 2004).

Cranial paraxial mesoderm provides a permissive substratum for the migrating CNCCs to populate the BAs. Studies using chick embryos suggest that cranial paraxial mesoderm is able to direct CNCC movement independently of their epithelial or mesenchymal organization (Noden 1986; Ferguson & Graham 2004). Of interest, cell fate mapping analysis has suggested that myoblast precursors contain positional identity inherited from their somatic mesenchymal stem cell precursors and can help to determine skeletal homologies that are based on muscle attachments (Matsuoka *et al.* 2005). Conversely, CNCCs, which provide most of the connective tissues and tendons in the head, may pattern and shape the individual cranial muscles (Noden 1986; Köntges & Lumsden 1996). In fact, it has been recently demonstrated that loss of TGF β signaling in CNCCs leads to decreased myogenic proliferation, reduced cell numbers, and disorganized tongue muscles (Hosokawa 2010). It has also been shown that CNCCs contain intrinsic information that can affect the entire facial patterning, although this contribution may depend upon the collective number of cells present at a given time and position (Schneider & Helms 2003; Tucker & Lumsden 2004).

Collectively, it is clear that craniofacial development requires continued interaction and contribution by cell populations derived from the ectoderm, the neural crest, the paraxial mesoderm, and the endoderm. The exchange of molecular information among tissues is essential for their patterning and fate determination. This operation

principle is crucial for both organogenesis and tissue regeneration.

Fate determination and differentiation of CNCC: the function of the TGF β -signaling pathway

The differentiation of NCCs along multiple distinctive pathways has generated considerable interest in developmental biology (Noden 1983; Tan & Morriss-Kay 1986; Bronner-Fraser 1993; LaBonne & Bronner-Fraser 1998). Controversy has existed as to whether *individual* NCCs are multipotent or whether their fates are restricted before they leave the neural tube or shortly afterward. Significant research has supported the idea that an important portion of the early neural crest population is composed of multipotent cells (Sieber-Blum & Cohen 1980; Baroffio *et al.* 1988; Bronner-Fraser & Fraser 1988, 1989; Rothman *et al.* 1990; Fraser & Bronner-Fraser 1991; Stemple & Anderson 1992; Shah *et al.*, 1996; Le Douarin & Dupin 2003). It has been suggested that, upon exposure to diverse environments, multipotent NCCs undergo progressive lineage restriction with their final determination dependent upon the instructions in a particular niche to support their survival, proliferation, and differentiation (Dorsky *et al.* 2000).

Conversely, it has also been proposed that particular neural crest subpopulations acquire a fate predisposition before or just after leaving the dorsal neural tube. This bias confers unique, cell-autonomous migratory properties on that particular subpopulation and allows those cells to access defined paths within the embryo (Schilling & Kimmel 1994; Erickson & Goins 1995; Henion & Weston 1997; Dorsky *et al.* 1998; Luo *et al.* 2003). Notably, a number of studies have demonstrated heterogeneity in the neural crest population, where both multipotent and restricted NCCs are able to coexist (Baroffio *et al.* 1988; Fraser & Bronner-Fraser 1991; Selleck *et al.* 1993; Le Douarin & Dupin 2003). The multipotent cells among the NCCs have been interpreted by some researchers as being representative of the neural crest stem cell (NCSC) population because they have the ability not only to give rise to diverse cell types but also to self-renew, a unique characteristic of stem cells (Stemple & Anderson 1992). Calloni and colleagues (2009) have demonstrated the existence of highly multipotent cells predominantly found in the cranial neural crest of quail embryos, which are able to produce clones comprising cell phenotypes as diverse as neurons, glia, melanocytes, chondrocytes, osteoblasts, and smooth muscle. However, migrating CNCCs contain relatively few NCSCs that are common to all of these cell types (Crane & Trainor 2006). Baroffio and colleagues (1991)

published similar findings showing that the majority of CNCCs give rise to clones composed of only one, two, or three distinct cell types, which identifies them as progenitor cells rather than stem cells (Crane & Trainor 2006).

In principle, environmental signals that influence NCSC or NCC progenitor fate might act through two distinct manners: a growth factor or signaling molecule could instruct a multipotent cell to acquire a particular fate at the expense of all other possible fates, or a signaling cue may select a specific cell lineage by supporting the survival of cells for a given stage and location or by specifically eliminating inappropriate cells (Sommer 2006). An example of the latter case is Shh, which favors the development of neural crest progenitors with skeletogenic and neurogenic potentials only and promotes chondrogenic differentiation (Calloni *et al.* 2007, 2009). A combination of stem cell factor (SCF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT-3) supports the melanocytic lineage (Sieber-Blum 1998). Similarly, endothelin3 (ET3) promotes proliferation and survival of melanocytic and glial progenitors (Lahav *et al.* 1998; Le Douarin *et al.* 2004), whereas FGF2 acts as a mitogen for NCSC (Zhang *et al.* 1997).

Several growth factors and other signaling molecules have been shown to act instructively on NCC progenitors and stem cells to promote specific cell types. For instance, constitutive expression of β -catenin by NCSC produces sensory neurons at the expense of virtually all other crest derivatives (Lee *et al.* 2004). Diverse members of the TGF β superfamily of growth factors are also expressed at sites where NCCs commit to a particular cell type and appear to be instructive for fate specification. For instance, BMP signaling has been shown to promote neurogenesis, whereas TGF β signaling favors smooth-muscle differentiation in the trunk NCCs (Shah *et al.* 1996). In contrast, CNCCs react to TGF β signaling differently. TGF β controls the differentiation of CNCCs to glial cells, and TGF β , BMP, and Wnt together control chondrocyte differentiation. The difference in responsiveness to growth factors between CNCCs and trunk NCCs might be due to the expression of Hox genes (Abzhanov *et al.* 2003; Creuzet *et al.* 2005). Interestingly, deletion of SMAD4, a mediator of both TGF β and BMP signaling pathways, in the neural crest affects sensory neurogenesis in the trigeminal ganglia and the survival of smooth muscle and proliferation of autonomic and ENS neuronal progenitor cells (Büchmann-Møller *et al.* 2009). Furthermore, the function of TGF β in regulating NCC differentiation is sensitive to the TGF β expression level, depending on whether it promotes alternative cell fates or induces apoptosis (Hagedorn *et al.* 2000).

One mechanism of TGF β influence in cell fate decisions is via the regulation of transcription factor expression (Shah *et al.* 1996; Dorsky *et al.* 2000). For example, the expression patterns of Tgf β 2 and the transcription factor MSX1 overlap during early stages, when CNCC-derived cells become specified to form dental and palatal mesenchyme, suggesting an epistatic relationship between these two genes (Ito *et al.* 2003). Compromised TGF β signaling affects the expression of MSX1, which in turn controls cell cycle progression of the CNCCs by regulating cyclin D1 expression (Ito *et al.* 2003). In vitro studies also suggest that MSX1 gene expression maintains cyclin D1 expression and keeps cells in an undifferentiated state by promoting proliferation (Hu *et al.* 2001). Other studies show that MSX1 might be involved in the maintenance of progenitor cells in the undifferentiated state and might induce cellular multipotency (Akimenko *et al.* 1995; Simon *et al.* 1995; Woloshin *et al.* 1995; Odelberg *et al.* 2000). In addition, MSX1 is involved in the development of CNCC-derived intramembranous bone, such as the frontal bone and the mandible. MSX1 expression is reduced in the osteogenic front of the mandibular bone and the mesenchymal cells between Meckel's cartilage and the mandibular bone in Tgfbr2;Wnt1-Cre mice. This finding suggests that TGF β signaling regulates osteoblast proliferation by controlling MSX1 expression during mandibular development (Oka *et al.* 2007).

Another transcription factor, dHAND, is an important member of the network of transcriptional regulators involved in NCC-derived sympathetic neuron development and is a downstream effector of BMP signaling (Howard *et al.* 2000). Both dHAND and eHAND are transcription factors that regulate determination and differentiation of progenitor cells as they give rise to skeletal myocytes, neurons, and hematopoietic cells (Shivdasani *et al.* 1995; Thomas *et al.* 1998; Firulli 2003). Significantly, the first BA ectoderm secretes a signal to stimulate the mesenchymal expression of dHAND, which can regulate MSX1 expression, suggesting that this signaling pathway plays an important regulatory role during craniofacial morphogenesis. Complete disruption of this molecular signaling pathway leads to growth failure of the BAs due to cell death, whereas partial disruption results in defects of BA derivatives, similar to those seen in CATCH-22 syndrome, including cardiac and facial defects (Wilson *et al.* 1993; Thomas *et al.* 1998).

Other examples of NCC fate specification induced by specific transcription factors regulated by members of the TGF β superfamily occur in the mandible and maxilla. During endochondral ossification of the proximal region of the mandible, TGF β signaling controls CNCC differentiation through the regulation of diverse chondro-

cytic and osteoblastic transcription factors. From the analysis of the angular region of the mandible in Tgfbr2;Wnt1-Cre mice, it has been proposed that a TGF β signaling pathway induces Sox9 and inhibits Runx2 and/or Dlx5 expression to direct osteochondroprogenitor cells into a chondrogenic lineage because there is a switch from chondrocytic to osteoblastic cell fate in CNCC-derived mesenchymal cells in this region of the mandible (Oka *et al.* 2007, 2008). Moreover, in the maxilla of Tgfbr2;Wnt1-Cre mice, there is a decrease in cell proliferation of CNCC-derived mesenchyme associated with upregulation of FoxO4 and Jun-B and a consequent reduction in cyclin D1 and D3 expression. Osteopontin/Spp, osteocalcin, osteonectin, collagen type I, and Runx2 are upregulated, suggesting that osteogenic differentiation is also accelerated in the maxilla, which is consistent with the reduction in size of this structure (Iwata *et al.* 2010). In contrast, the basal transcription factor Taf4b is downregulated in the maxilla of these mutants. Taf4b and Taf1 mediate TGF β signaling activity that promotes CNCC proliferation and osteogenic differentiation (Iwata *et al.* 2010). Taken together, TGF β signaling mediates downstream transcription factors to play a critical role in the regulation of and balance between proliferation and differentiation of CNCC-derived tissues and organs.

Stem cell properties of CNCCs and their potential for alveolar bone regeneration

Among many tissues that originate from CNCC, tooth and alveolar bone have an interdependent relationship. Alveolar bone provides the support for a functional dentition, and it can be reshaped based on the precise needs of the tooth. Development of the alveolar bone involves CNCC-derived mesenchymal cell condensation to form the dental sac, osteoid deposition, and mineralization. A well-defined intramembranous bony socket, consisting of differentiating osteoblasts that are enriched in bone matrix, is built surrounding the developing tooth. Clinically, loss of a tooth leads to the absorption of alveolar bone, suggesting that there is a close relationship between alveolar bone and tooth development (Zhang *et al.* 2003). Therefore, to design a biological solution for tooth regeneration, it is critical to regenerate alveolar bone that is fully integrated with and provides support for the newly formed tooth (Chung *et al.* 2009). To do so, it is necessary to understand the way in which CNCCs differentiate into osteoblasts and the molecular mechanisms that regulate the formation of alveolar bone. Recently an in vitro culture system for CNCCs was developed to understand the proliferation and differentiation potential of postmigratory CNCCs within the

first BA through the analysis of Wnt1-Cre;R26R mouse embryos. Specifically, cells from embryonic days 9.5 and E10.5 were studied because they provide undifferentiated CNCCs (Zhao *et al.* 2006). This study demonstrated a robust proliferative capability of pure CNCCs along with maintenance of their undifferentiated state. Specific culture conditions lead to their differentiation into particular cell types, faithfully mimicking the differentiation process of postmigratory CNCCs *in vivo*, including neurons, Schwann cells, myofibroblasts, and, more interestingly, osteoblasts (Zhao *et al.* 2006).

Recently, Chung and colleagues (2009) have also demonstrated that postmigratory CNCCs maintain stem cell characteristics into adulthood. These cells express mesenchyme stem cell (MSC) markers, such as CD90.2 and SSEA4, which are also expressed by embryonic stem cell (ESC) lines (International Stem Cell Initiative *et al.* 2007). CNCCs share more characteristics with MSCs and ESCs than with bone marrow mesenchymal stem cells (BMMSCs). Interestingly, the expression pattern of some MSC markers is modified during development. In MSCs from the mandible of adult mice, the expression level of CD90.2 and SSEA4 decreased and that of CD29, CD44, Sca-1, and CD49e increased compared with MSCs from embryonic stages. Therefore, properties of CNCC-originated stem cells change during their contribution to the development of craniofacial structures (Chung *et al.* 2009). Postmigratory CNCCs are more responsive to *in vitro* osteogenic induction than BMMSCs, consistent with previous findings showing that neural crest-derived progenitor cells possess increased osteogenic capacity and enhanced osteogenesis compared with mesoderm-derived progenitor cells (Leucht *et al.* 2008). In addition, following transplantation into hosts, CNCCs are able to form bone with densely packed lamella structures that are separated by abundant connective tissues. Unlike bone originating from BMMSCs, CNCC-derived bone does not contain prominent hematopoietic components (Chung *et al.* 2009), which is characteristic of craniofacial bone from intramembranous ossifications. The differences in the histological appearance of bone formed by postmigratory CNCCs and BMMSC might be the result of intrinsic differences in embryological origin and functional demands at each skeletal site (Akintoye *et al.* 2006). For instance, long bones are physiologically adapted to support body weight, contain more bone marrow, and contribute more to hematopoiesis (Charbord *et al.* 1996). In contrast, CNCC-originated mandible and maxilla are parts of the craniofacial complex and contain less bone marrow, but offer protection to vital structures including paranasal sinuses, dentition, and neurovascular bundles (Akintoye *et al.* 2006).

Skeletal bone formation results from endochondral ossification, in which hypertrophic chondrocytes mineralize their surrounding matrix and attract blood vessels. Consequently, long bones contain more abundant bone marrow than craniofacial bones. The proper formation of bone marrow requires the normal development of skeletal bones. Several studies of mutant mice, in which hematopoiesis is defective as a consequence of primary defects in bone development, have implicated osteoblasts in the formation and function of the bone marrow hematopoietic stem cell environment (Wilson & Trumpp 2006). In addition, cells involved in bone formation play a role in supporting hematopoiesis, and specialized osteoblasts lining the bone marrow function to maintain and regulate hematopoietic stem cells (Wilson & Trumpp 2006). During endochondral ossification, hypertrophic chondrocytes express BMP2 and BMP6. Deficiency of these two genes in mice results in reduction of trabecular bone volume along with suppressed bone formation (Kugimiya *et al.* 2005; Wilson & Trumpp 2006). Accordingly, BMP2 induces increased bone marrow along with increased bone formation in transplants of CNCCs and hydroxyapatite/tricalcium phosphate. Thus, the concerted action of BMP2 and CNCCs results in increased formation of hematopoietic components in the bone matrix, consistent with previous studies showing that bone marrow development in the rat mandible occurs in a BMP2 dose-dependent fashion (Arosarena & Collins 2005). These findings support the hypothesis that the microenvironment influences how postmigratory CNCCs differentiate and regenerate tissues. Clinically, BMP2 has been widely used to promote jawbone formation in order to support restoration (Seto *et al.* 2002; Herford & Boyne 2008).

Postmigratory CNCCs may be crucial for tooth germ survival. Well-formed supporting structures, especially the alveolar bone, are also required for the proper development of teeth. Subcutaneous transplantation of tooth germs with postmigratory CNCCs results in apparently normal tooth development (Chung *et al.* 2009). CNCCs contributed to the dental pulp and the bone adjacent to the tooth. In contrast, transplants of BMMSCs and tooth germs failed to form a normal tooth and the supporting structures were altered. Thus, it appears that CNCC-derived bone, with features of craniofacial bone, is required for the survival of tooth germs. Differences in supporting structures based on differences in embryonic origin between CNCCs and BMMSCs might explain the abnormal tooth development in BMMSC-tooth transplants (Chung *et al.* 2009). Various transcription factors and signaling molecules, including BMP, FGF, Activin, Hedgehog, and Wnt family members, participate in tooth development (Thesleff & Sharpe 1997; Peters &

Balling 1999). Among these, BMPs are key signals (Vainio *et al.* 1993; Plikus *et al.* 2005). During jawbone development, BMP activity plays a vital role in the formation of alveolar bone via a BMP/MSX signaling cascade (Zhang *et al.* 2003; Nie *et al.* 2006; Zhao *et al.* 2000). In loss-of-function studies that inhibit BMP activity using Noggin, kidney capsule transplantation of Noggin-treated tooth germs gave rise to keratinized cysts (Zhang *et al.* 2003). Similarly, inactivation of SMAD4 in CNCCs results in abnormal tooth development in tooth germ transplants. However, administration of BMP4 into transplants of BMMSC and tooth germs failed to promote normal tooth development (Chung *et al.* 2009), suggesting that BMP signaling in CNCCs is required but not sufficient to support tooth formation. Remarkably, there is an intrinsic difference between CNCCs and BMMSCs in tooth development; postmigratory CNCCs have unique properties essential for tooth development. Postmigratory CNCCs create a link between alveolar bone and tooth development as a functional unit. These cells have the ability to support an organ survival environment, also known as an *organ niche*, which can provide the proper niche conditions for tooth germ survival.

Identification of mesenchymal stem cells in the craniofacial region

As implied earlier, regeneration of a functional and living tooth, periodontal tissue, and bone is one of the most promising therapeutic strategies for the replacement of diseased or damaged tissue in humans. It has been proposed that cell-based strategies show promising potential for regeneration in other mammals (Chai & Slavkin 2003; Duailibi *et al.* 2004; Ohazama *et al.* 2004; Yen & Sharpe 2006). To date, several populations of human multipotent mesenchymal stem cells have been isolated from a variety of dental or orofacial tissues. These include dental pulp-derived dental pulp stem cells (DPSCs; Gronthos *et al.* 2000), stem cells from human exfoliated deciduous teeth (SHED; Miura *et al.* 2003), periodontal ligament-derived stem cells (PDLSCs; Seo *et al.* 2004), root apical papilla-derived stem cells (SCAPs; Sonoyama *et al.* 2006), dental follicle-derived progenitors (Morsczeck *et al.* 2005), and gingival tissue-derived MSCs (GMSCs; Zhan *et al.* 2009). Recently, tooth structures such as dentin, dental pulp, cementum, periodontal ligament, and bio-root have been regenerated in murine and swine models using stem cell-based regenerative approaches (Gronthos *et al.* 2000, 2002; Seo *et al.* 2004; Sonoyama *et al.* 2006; Liu *et al.* 2008; Huang *et al.* 2010). Additionally, SHED was found to be able to form significant amounts of bone when transplanted into immunocompromised mice subcutaneously, providing a

new cell source for alveolar and orofacial bone regeneration (Miura *et al.* 2003; Seo *et al.* 2008; Zheng *et al.* 2009). Owing to the complexity of human tooth growth and development, the regeneration of a whole tooth structure, including enamel, dentin–pulp complex, and periodontal tissues, as a functional entity in large animal models or humans has not yet been successful. To achieve functional tooth regeneration, it is necessary to further understand the interplays among tissue specific stem cells, surrounding host tissue and cellular environments, and recipient immune responses.

Dental pulp stem cells (DPSCs)

DPSCs were isolated from digested dental pulp tissue by a single colony selection using an immunomagnetic method with anti-stromal-derived factor 1 (STRO-1) antibody. These cells exhibited clonogenic cell cluster, highly proliferative characteristics, in vivo self-renewal, multiple differentiations in vitro, and typical immunoreactivity profile as seen in bone marrow mesenchymal stem cells (Gronthos *et al.* 2000). One of the most important advances in DPSC study was the revelation of their stem cell niche in the perivascular region (Shi & Gronthos 2003; Shi *et al.* 2005). Extensive immunophenotyping of ex vivo-expanded DPSCs demonstrated their expression of various markers associated with endothelial and/or smooth muscle cells, such as STRO-1, vascular cell adhesion molecule 1 (VCAM-1), melanoma-associated antigen/mucin 18 (MUC-18), and smooth muscle-actin (Gronthos *et al.* 2000). In addition, smooth muscle-actin-positive cells have also been detected close to mineralized deposits in human dental pulp cultures (Alliot-Licht *et al.* 2001). It is expected that further characterization of DPSCs by current molecular technology will provide novel markers for purifying and identifying unique subsets of DPSCs.

To explore tissue regeneration potential, ex vivo expanded DPSCs were implanted into immunocompromised mice subcutaneously using hydroxyapatite–tricalcium phosphate (HA/TCP) as a carrier. It was found that DPSCs are capable of forming ectopic pulp–dentin-like complexes in vivo (Gronthos *et al.* 2000; Batouli *et al.* 2003). This ectopic dentin–pulp-like tissue is surrounded by a layer of odontoblast-like cells expressing dentin sialophosphoprotein (DSPP) and forming dentinal tubule-like structures as seen in natural dentin. Moreover, when DPSCs were seeded onto human dentin surfaces and implanted into immunocompromised mice, reparative dentin-like structure was deposited on the dentin surface (Batouli *et al.* 2003). Furthermore, a recent study indicated that pulp-like tissue can be regenerated de novo in emptied root canal space by

DPSCs and SCAPs as evidenced by odontoblast-like cells producing dentin-like tissue on existing dentinal walls (Huang *et al.* 2010). These data suggest a potential for using dental stem cells to regenerate pulp tissue. Very recent studies showed successful generation of induced pluripotent stem (iPS) cells from dental stem cells including DPSCs, SHED, and SCAPs (Tamaoki *et al.* 2010; Yan *et al.* 2010), providing the opportunities to investigate early developmental characteristics of dental stem cells and perhaps using these dental iPS cells for regenerative purpose. However, whether these dental iPS cells retain specific dental origin characteristics is unknown.

Stem cells from human exfoliated deciduous teeth (SHED)

The transition from deciduous teeth to adult permanent teeth is a very unique and dynamic process in which the development and eruption of permanent teeth coordinate with the resorption of the roots of deciduous teeth. Stem cells from SHED have been isolated from naturally exfoliated deciduous teeth with the capacity to differentiate into osteogenic and odontogenic cells, adipocytes, and neural cells (Miura *et al.* 2003). As neural crest cell-associated postnatal stem cells, SHED express a variety of neural cell markers including nestin, beta III tubulin, GAD, NeuN, GFAP, NFM, and CNPase (Miura *et al.* 2003). It was reported that SHED were able to form bone when transplanted into immunocompromised mice (Miura *et al.* 2003; Laino *et al.* 2006) and offered optimal bone regeneration for repairing craniofacial bone defects in mice (Seo *et al.* 2008) and swine (Zheng *et al.* 2009). SHED also were able to differentiate into functional odontoblast and angiogenic endothelial cells (Sakai *et al.* 2010). More interestingly, SHED were found to show an improved therapeutic effect for ameliorating systemic disease through reconstructing a balance of regulatory T cells and Th17 cells in systemic lupus model mice (Yamaza *et al.* 2010) analogous to the BMMSC therapy (Sun *et al.* 2009). Taken together, SHED has unique properties for tissue regeneration and immune modulation.

Stem cells from apical papilla (SCAP)

Reconstruction of teeth in patients without adequate bone support would be a major advance. Stem cell-mediated root regeneration offers opportunities to regenerate a bio-root, and it is associated with periodontal and bony tissues, which are necessary for maintaining the physiological function of teeth. In order to isolate SCAP, detached apical papilla tissue was digested by collagenase and dispase and subsequent culture showed the formation of single colony clusters (Sonoyama *et al.*

2006). This colony-forming cell population showed a high proliferation rate and high expression levels of survivin and telomerase when compared to DPSCs derived from the same tooth. The immunophenotype of SCAP is similar to that of DPSCs in terms of expression of surface molecules. In addition, SCAP showed widely expressed neurogenic markers, such as nestin and neurofilament M, and also showed an elevated *in vivo* dentin regeneration capacity versus DPSCs (Sonoyama *et al.* 2006, 2008).

Identification of SCAP provides an opportunity to improve *in vivo* dentin regeneration. To play a functional role, the root has to connect with the periodontal ligament to ensure correct positional stability and support tooth function. In order to regenerate bio-root and PDL tissue, SCAP and PDLSCs were combined for dentin and PDL regeneration. The root shape HA/TCP block containing SCAP coated with Gelfoam containing PDLSCs was implanted into the extracted tooth socket and sutured for three months. CT examination revealed a HA/SCAP-Gelfoam/PDLSC structure growing inside the socket with mineralized root-like tissue formation and periodontal ligament space. After a cemented porcelain crown, bio-roots were able to support the crown and function for four weeks. Newly formed bio-roots showed a significantly improved compressive strength than that of original HA/TCP carriers after six months of implantation. These findings suggest the feasibility of using a combination of SCAP/PDLSCs in conjunction with artificial crowns for functional tooth regeneration. The bio-root was comprised of dentin and pulp-like structures encircled with periodontal ligament tissue (Sonoyama *et al.* 2006).

Periodontal ligament stem cells (PDLSCs)

The periodontal ligament (PDL) is a unique connective tissue between the cementum and inner wall of the alveolar bone socket. The important role of PDL is not only supporting teeth, but also contributing to tooth nutrition, homeostasis, and repair of damaged tissue. Multipotent mesenchymal stem cells in PDL (periodontal ligament stem cells, or PDLSCs) were identified in 2004 (Seo *et al.* 2004). These PDLSCs showed clonogenic cell clusters and capacity for developing into adipocytes, osteoblast- and cementoblast-like cells *in vitro*. Moreover, PDLSCs were capable of forming cementum- and periodontal ligament-like tissues *in vivo* (Seo *et al.* 2004; Shi *et al.* 2005; Gronthos *et al.* 2006). PDLSCs shared several surface markers, including STRO-1 and CD146/Muc18, with bone marrow mesenchymal stem cells (BMMSCs; McCulloch & Melcher 1983, McCulloch & Bordin 1991; Seo *et al.* 2004). The comparison between PDLSCs and BMMSCs suggests that PDLSCs represent

a MSC population derived from PDL tissue. A recent swine study showed that periodontal disease could be recovered by implantation of autologous and allogenic PDLSCs (Liu *et al.* 2008; Ding *et al.* 2010). The autologous PDLSCs were transplanted into the surgically created periodontal defect in swine. New bone, cementum, and periodontal ligament were regenerated in the periodontal defect area in the PDLSC-treated group, and the height of the new alveolar bone was greater in the PDLSC treated group than that in carrier treated group. Histopathological photomicrography also showed newly generated bone and periodontal tissues in the PDLSC treatment group. In addition to forming cementum and periodontal ligament, PDLSCs were able to regenerate Sharpey's fibers anchored into the newly regenerated cementum (Liu *et al.* 2008). In a human clinical pilot study, autologous periodontal ligament progenitor (PDL) cells were implanted into deep intra-bony defects with a probing depth (PD) $\geq$ 6 mm. After 32–72 months post implantation, clinical examination indicated that transplantation of PDLs provides a stable long-term therapeutic improvement and remarkable periodontal tissue regeneration. All treated patients showed no adverse effects during follow-up (Feng *et al.* 2010).

Immunomodulatory property of dental or orofacial mesenchymal stem cells

A growing body of evidence indicates that BMSCs produce a variety of cytokines and display profound immunomodulatory properties (Nauta & Fibbe 2007; Uccelli *et al.* 2007, 2008) by inhibiting the proliferation and function of several major immune cells such as natural killer cells, dendritic cells, and T and B lymphocytes (Aggarwal & Pittenger 2005; Nauta & Fibbe 2007; Uccelli *et al.* 2007, 2008). These unique properties cause MSCs to be of great interest to researchers searching for clinical applications in treating immune disorders (Nauta & Fibbe 2007; Bernardo *et al.* 2009). Early experimental evidence demonstrated that DPSCs, SCAPs, SHED, PDLSCs, and GMSCs possess immunomodulatory properties. DPSCs showed 91.4% inhibition of PHA-activated T cell response as assessed by a 3H-thymidine assay (Pierdomenico *et al.* 2005). Both freshly isolated SCAPs and cryopreserved SCAPs showed similar potential for inhibiting PHA-stimulated T cell activity (Ding *et al.* 2010). SHEDs were found to show a significant effect in inhibiting differentiation of Th17 cells and promoting CD4⁺CD25⁺Foxp3⁺ regulatory T cells in vitro. Moreover, systemic infusion of SHEDs is capable of effectively reversing systemic lupus erythematosus in mice (Yamaza *et al.* 2010).

PDLSCs suppress the proliferation of activated peripheral blood mononuclear cells (PBMNCs) via soluble factors such as TGF β , HGF, and IDO that are partly dependent on IFN- γ synthesized by activated PBMNCs (Wada *et al.* 2009). GMSCs showed a capacity for immunomodulation, specifically suppressing peripheral blood lymphocyte proliferation and inducing expression of immunosuppressive factors including IL-10, IDO, inducible NO synthase (iNOS), and cyclooxygenase 2 (COX-2) in the presence of IFN- γ in vitro. In addition, systemic infusion of GMSCs in experimental colitis significantly ameliorated both clinical and histopathological severity of the colonic inflammation through suppression of inflammatory infiltrates and cytokines/mediators, and elevation of infiltration of regulatory T cells along with expression of anti-inflammatory cytokine IL-10 at the colonic sites (Zhan *et al.* 2009). This experimental evidence clearly indicates that dental or orofacial mesenchymal stem cells are promising cell sources for immunotherapies.

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Clinical correlate: stem cell therapy for craniofacial bone regeneration

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Current treatment modalities in the rehabilitation of oral and craniofacial tissues provide functional and structural restoration of the compromised or lost tissue; yet, many of these approaches do not meet the need for more biologic and physiologic treatment outcomes. Traditional therapies for craniofacial reconstructions typically employ the use of allografts, xenografts, and synthetic materials (Cochran 1996; Coulthard *et al.* 2003), but innovative cell- and tissue-based strategies are being developed to overcome the problematic limitations of these traditional treatments (Ohazama *et al.* 2004; Zaky & Cancedda 2009).

Though the emergence of cell therapy science has been a gateway to new paradigms of treatment for tissue regeneration, a major challenge in this new arena is the identification of the most appropriate cell source for use in these regenerative approaches. Recent evidence cites different cell types, of different origins, having “stem-like” properties and the capacity to regenerate a variety of craniofacial tissues including cartilage, bone, blood vessels, salivary gland, gingiva, and tooth tissues (Krebsbach & Robey 2002; Yan *et al.* 2006; Macchiarini *et al.* 2008; Delaere *et al.* 2010). Due to their osteogenic capacity in preclinical model systems, bone marrow-derived mesenchymal stem cells have gained much attention for use in cell-based tissue regenerative approaches (Krebsbach *et al.* 1997; Gao *et al.* 2001; Holtorf *et al.* 2005; Kaigler *et al.* 2006). More recently, there have been a few clinical reports investigating the potential of autologous grafts containing bone marrow-derived cells in the repair of skeletal and craniofacial defects. Though these preliminary reports hold great promise, there are two major limitations (Gimbel *et al.*

2007; Marcacci *et al.* 2007; Pelegri *et al.* 2010; Soltan *et al.* 2010) common to them all: (1) the techniques used for cell isolation and expansion are crude, and (2) the grafts and cells used for clinical transplantation are not well characterized.

Tissue repair cells (TRCs), developed by Aastrom Biosciences (Ann Arbor, MI, USA), represent an autologous, bone marrow-derived mixed-cell population containing mesenchymal stem cells, produced by an automated cell-manufacturing process utilizing a single-pass perfusion (SPP) system. In SPP, culture medium is continuously replaced by fresh medium at a slow, controlled rate without the disturbance, removal, or passaging of cells; this enables a clinical-scale expansion of TRCs to numbers not achievable through conventional culturing techniques. TRCs are characterized following their production, and these enriched CD90+ cell populations are highly reproducible (Dennis *et al.* 2007). Here, we report a case utilizing TRCs in an autologous cell therapy approach (Figure 11.1A) to reconstruct a localized osseous defect in the maxilla and restore with a dental implant.

Case presentation

This case was part of a larger randomized controlled clinical trial that was conducted over the course of 15 months. Following US Food and Drug Administration (FDA) and University of Michigan Institutional Review Board (IRB) approval, the patient underwent a bone marrow aspiration from the posterior ilium under conscious sedation and local anesthetic. Collected marrow was transferred to a sterile blood bag and bone marrow mononuclear cells (BMMNCs) were purified by Ficoll

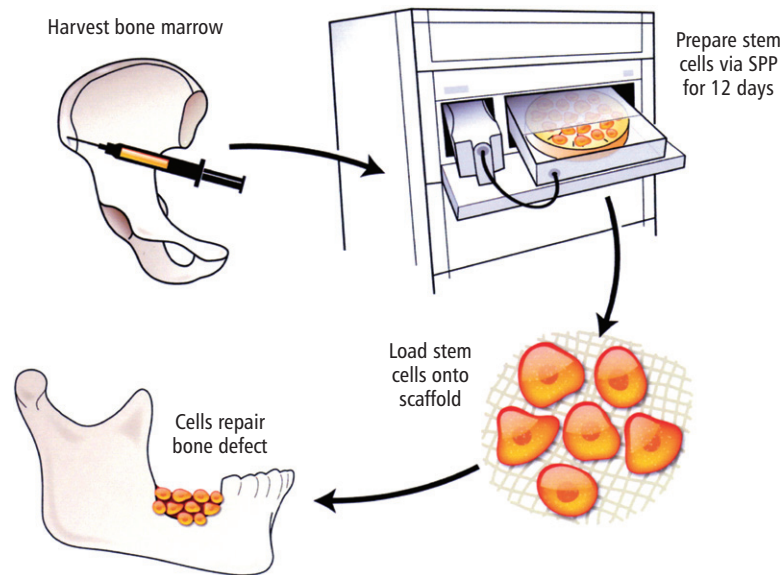


Figure 11.1 Cell therapy schematic. Cell therapy approach for the treatment of jawbone defects. (Reprinted from Kaigler *et al.*, 2010, with permission from Mary Ann Liebert, Inc.)

density gradient centrifugation. BMMNCs were then inoculated into an Aastrom Replicell® System bioreactor, which is a proprietary, computer-controlled, automated cell-processing unit. The cell cassette within this system provides a functionally closed, sterile environment in which cell production occurs. The fluid pathway in the cell cassette includes the cell growth chamber, a medium supply container, a mechanism for medium delivery, a waste medium collection container, and a container for the collection of harvested cells. This system incorporates SPP in which fresh medium flows slowly over cells without retention of waste metabolites or differentiating cytokines. After cultivation for 12 days at 37°C and 5% CO₂, the TRC product was harvested, washed, collected into a sterile bag, and stored until the time of surgical transplantation (Figure 11.2A). FACs analysis was performed on the TRCs following their production and prior to transplantation. Figure 11.2B shows collection of cells into a 5 cc syringe for loading (Figure 11.2C) of 2 cc of 1.5×10^7 cells/cc cell suspension onto a gelatin sponge scaffold ($5 \times 15 \times 20$ mm) to the point of saturation of the sponge (Figure 11.2D). Cells were allowed 15 minutes to adhere to the gelatin sponge prior to their placement into the osseous defect site. Scanning electron microscopy (SEM) shows the distribution of cells within the internal aspect of the sponge just prior to transplantation, and confirms that cells were distributed throughout the sponge and tended to adhere in large clusters (Figure 11.2E).

The osseous defect to which the cell therapy was applied was created following tooth extraction of a frac-

tured nonrestorable tooth (Figure 11.3A–B). After the extent of the bone defect was assessed (Figure 11.3C–D), the sponge matrix containing the TRCs was grafted into the bone defect (Figure 11.3E). The flap was then coronally repositioned, and a bioabsorbable collagen barrier membrane (Biomend®, Zimmer Dental, Carlsbad, CA, USA) was placed over the sponge for containment of the cells. The tissues were then approximated and closed with a 4-0 bioabsorbable suture material (Vicryl, Ethicon, Somerville, NJ, USA). Oral hygiene instructions included 0.12% chlorhexidine mouth rinses and no brushing in the area of the graft for two weeks to reduce risk of oral infection and membrane exposure. The patient was prescribed oral antibiotics, Amoxicillin 500 mg, taken every eight hours for seven days and an anti-inflammatory and pain reliever medication, Ibuprofen 600 mg, taken every six hours for three days. Surgical re-entry of the treated osseous defect was performed six weeks after surgery (Figure 11.3G) and a bone core biopsy of 2×7 mm was harvested with a trephine drill (Figure 11.3H). The bone core was immediately prepared for micro-CT (μ CT) imaging and descriptive histological evaluation. A dental implant was placed at the time of biopsy harvest and restored with an implant supported crown six months later (Figure 11.3I–J).

Upon retrieval of the bone core biopsy specimen with the trephine drill, it was found that the tissue was very dense, indicative of mature bone tissue (Figure 11.4A). The clinical appearance of the biopsy specimen clearly showed a highly vascular tissue that, consistent with retrieval of viable bone tissue, produced bleeding follow-

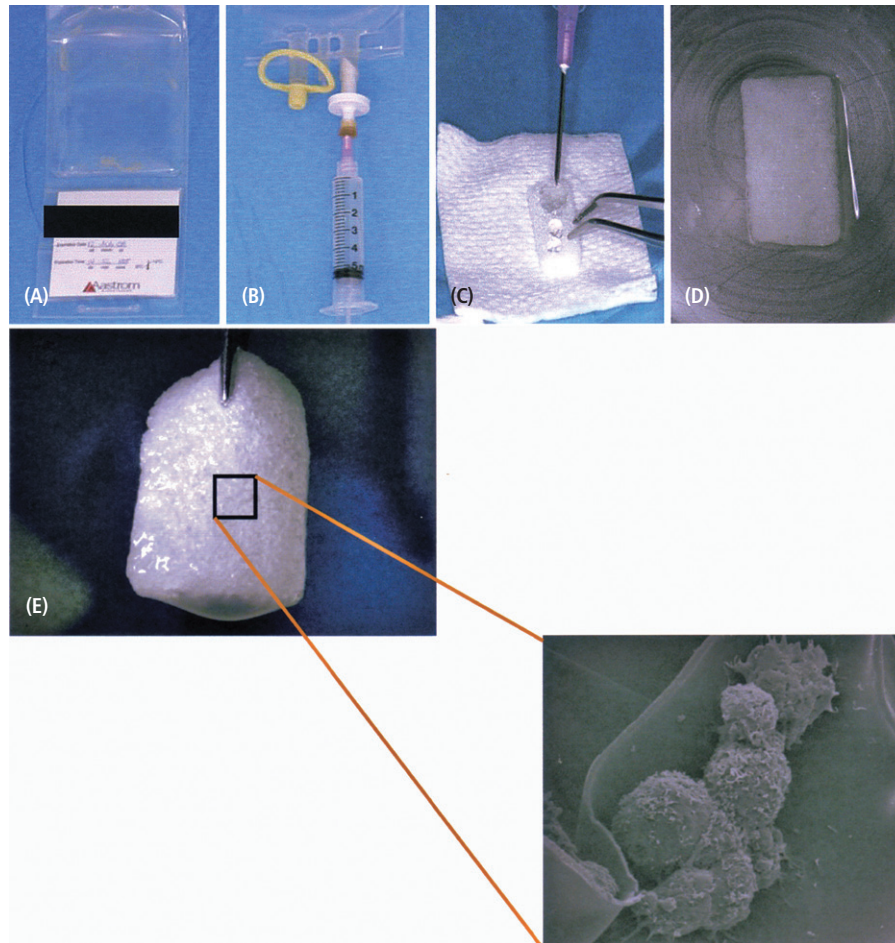


Figure 11.2 Cell preparation for cell delivery. A. Cell packaging following production of bone repair cells (BRCs). B. Collection of BRCs into syringe for loading. C. Loading of gelatin sponge with BRC suspension. D. Saturation of gelatin sponge with 2 ml of 1.5×10^7 cells/ml suspension. E. SEM of cells within the interior aspect of the gelatin sponge 15 minutes following loading.

ing harvest (Figure 11.4B). μ CT analysis was performed on the biopsied tissue and three-dimensional (3D) reconstruction of the specimen showed highly mineralized tissue throughout the entire length of the core (Figure 11.4C). Following this analysis, histology was performed on the specimen and Masson's trichrome staining clearly showed widespread distribution of mature bone tissue with an abundance of blood vessels distributed throughout (Figure 11.4D). These results provide the first published evidence that TRCs, expanded from a small amount of BMMNCs, have the regenerative capacity to produce highly vascular bone tissue in a human craniofacial bone defect.

Discussion

Cell transplantation of stem cells has tremendous potential for craniofacial regenerative applications, yet identification of the appropriate cell types and cell-processing

protocols are two of the most critical determinants in producing successful outcomes. Though many *in vitro* and *in vivo* studies have been conducted to examine the phenotype and regenerative potential of different stem and progenitor cell populations, the ultimate test of cell therapies lies in the clinical regenerative potential. To address this clinical situation, we examined the therapeutic potential of autologous bone marrow-derived stem cells, TRCs, in an osseous defect of the jawbone.

The field of regenerative medicine aims to use tissue engineering and biomimetic strategies to functionally restore and replace damaged and lost tissue (Langer & Vacanti 1993). The extraction socket created following tooth removal serves as a good model of human bone regeneration in that it is highly reproducible and yet has a limited capacity to regenerate without intervention. In a recent clinical study involving 13 subjects, Pelegrine and colleagues (2010) used the human extraction socket as a model to investigate the potential of a bone marrow

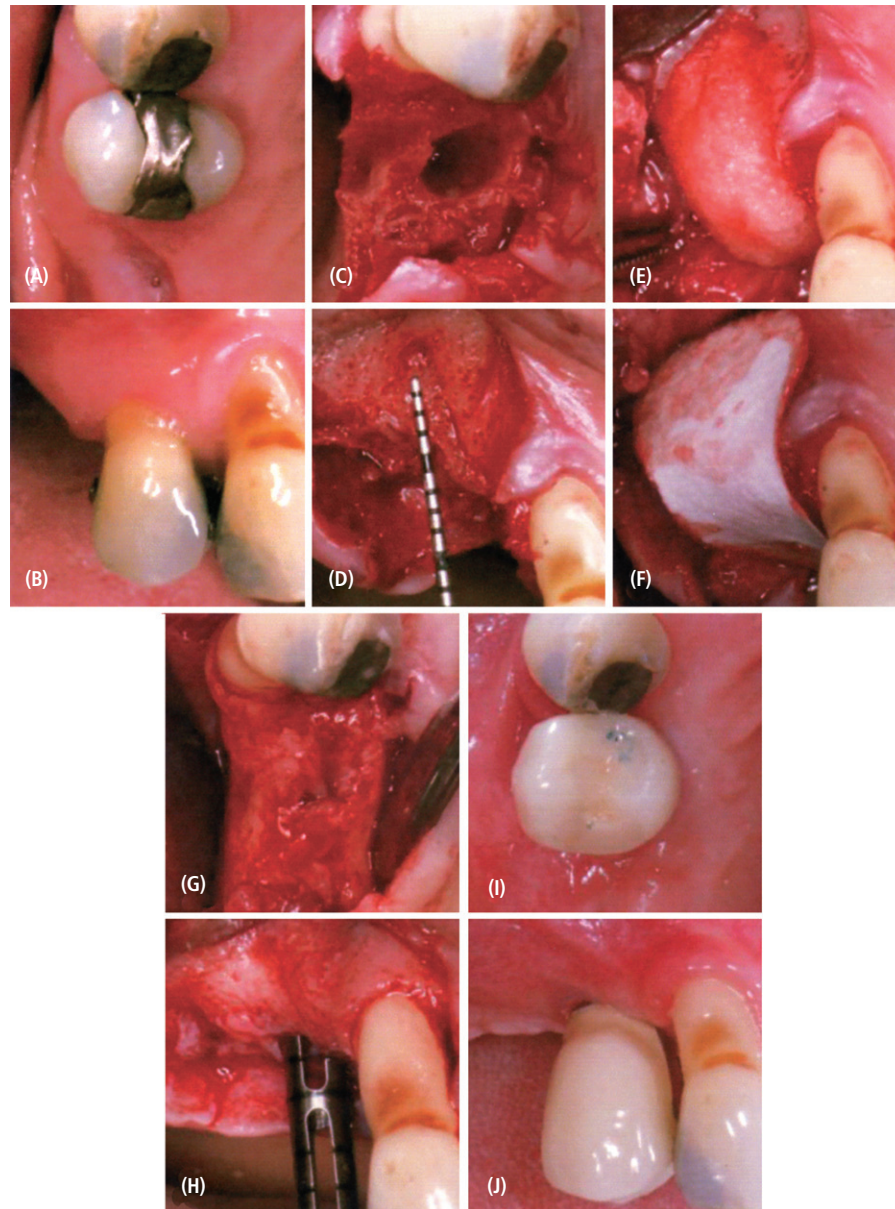


Figure 11.3 Clinical cell therapy. A. and B. Nonrestorable tooth just prior to removal. C. Osseous defect created following tooth removal. D. Measurement of extent of the defect with a periodontal probe. E. Sponge placement within defect. F. Placement of barrier membrane for cell containment. G. Regenerated osseous defect after six weeks. H. Harvesting of bone core biopsy. I–J. Restored tooth six months following placement of dental implant. (Reprinted from Kaigler *et al.*, 2010, with permission from Mary Ann Liebert, Inc.)

graft in preserving alveolar bone following tooth extraction (Pelegrine *et al.* 2010). Upon re-entry into these sites at six months, they demonstrated that the bone marrow graft provided better results in maintaining the alveolar ridge than when no graft was used. The results suggested that bone marrow constituents hold bone regenerative potential, yet no characterization of the cellular component of the graft was conducted. In addition, these grafted sites were re-entered for harvesting of regenerated tissue and histological evaluation six months after grafting.

Other promising preliminary reports of successful craniofacial regenerative procedures using bone marrow-derived grafts are similarly confounded by the lack of characterization of the grafted material (Filho Cerruti *et al.* 2007; Meijer *et al.* 2008; Soltan *et al.* 2010). Not having an understanding of what the starting product is in these procedures makes it difficult to draw conclusions, particularly if the results aimed for are not achieved. A general critique of cell therapy approaches has been the lack of reproducible cell isolation, expansion, and

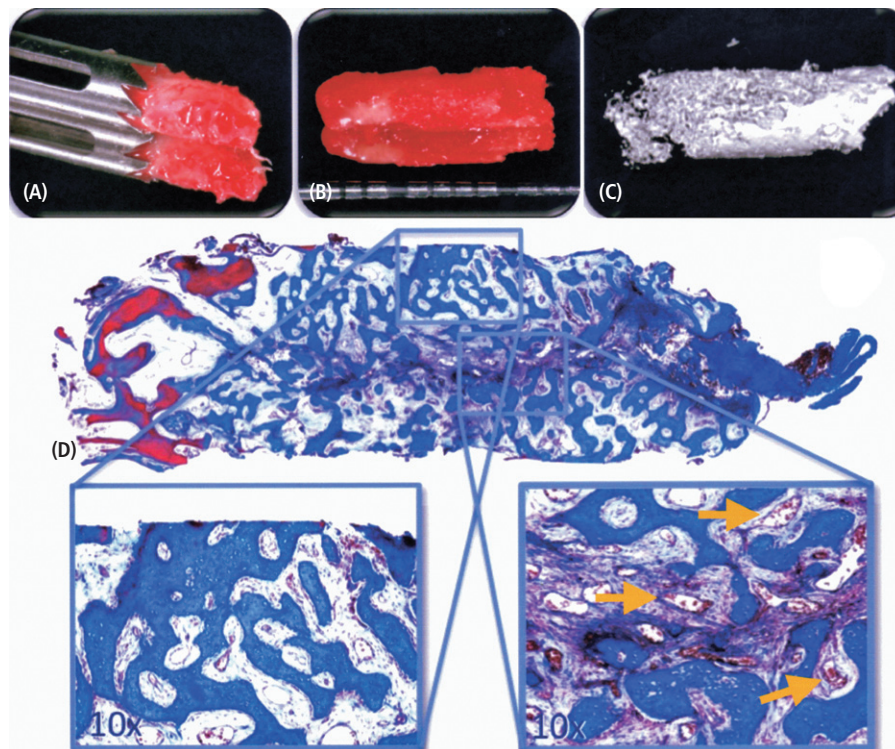


Figure 11.4 Six-week μ CT and histological analyses. A–B. Bone core biopsy of bone regenerated with cell therapy. C. Three-dimensional reconstruction of regenerated bone core biopsy. D. Histological evaluation (Masson's trichrome staining) of bone formation showing areas of mature cortical bone with high vascularity, as indicated by the abundance of blood vessels (arrows).

processing protocols, which can predictably yield consistent cell populations. Through the TRC processing protocol used to produce TRC in our study, within a given range, there is a relative degree of homogeneity between patients in the final TRC population that is produced. The SPP process for generation of TRC populations maintains consistent results and this is ensured through cell surface marker characterization of all TRC populations prior to use of these cells (Dennis *et al.* 2007; Kaigler *et al.* 2010). Along these lines, in cell therapy approaches, initial characterization of the transplanted cell type(s) may enable more targeted therapeutic interventions.

Despite the promising results of this case report, it is important to note that these results were obtained in a single patient and thus limit any general conclusions that can be drawn. This case presentation is part of a larger, FDA-regulated, randomized, controlled Phase I/II trial where a larger number of patients have been treated with TRC in a similar type of defect. This larger study is ongoing and it includes a one-year patient follow-up; however, upon study completion, all of the clinical data will be analyzed and the results outlined in a future report. While it is understood that the feasibility of this protocol for routine tooth extraction surgeries is most likely not practical, this study was conducted as an FDA

Phase I/II study to examine safety and efficacy of this therapy for regeneration of craniofacial bone. To this end, it should be recognized that cell-based therapies require navigation through a rigorous regulatory process before they can be studied clinically, and certainly before they can be practiced widely (Caunday *et al.* 2009; Zaky & Cancedda 2009). Nonetheless, additional clinical investigations are certainly warranted and currently underway as the therapeutic potential for these therapies is very promising.

Conclusion

There is a growing interest in cell therapy strategies to regenerate craniofacial tissues, particularly bone. However, critical questions to be considered in using these strategies are: what is the source of cells used in these approaches? How will the cells be processed and expanded to reach clinical-scale numbers for application? What are the phenotypes and regenerative capacities of the cells produced? Though the case presented herein does not provide the answers to the aforementioned questions, it does provide insight toward the clinical regenerative potential of craniofacial cell therapy.

Acknowledgments

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Extracellular matrix and mineralization of craniofacial bone

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Specialized connective tissues such as bones (including cartilage) and teeth (excluding enamel) are distinct from other connective tissues not only because they contain mineral but also because the collagen contained therein is cross-linked in different ways and is integrated with a unique set of additional extracellular matrix proteins specific to these tissues. Collectively, the proteins and proteoglycans of the extracellular matrix of bone provide an appropriate scaffolding to accommodate billions (if not trillions in the entire skeleton) of nanosized calcium-phosphate apatitic mineral crystals, and the noncollagenous proteins are widely thought to regulate the mineralization process (McKee *et al.* 2005). Bone extracellular matrix is generally similar in composition and structure throughout the skeleton, including the various skeletal elements of the craniofacial complex, although some variations can exist across different anatomical sites and across different ages. Generally speaking, embryonic and fetal bone is considered to be “woven” in nature, with more or less randomly interwoven collagen fibrils being less densely packed and with a greater abundance of noncollagenous proteins and small proteoglycans than the denser, more oriented collagen fibrils appearing postnatally as orthogonal lamellar arrays. Bone extracellular matrix is imbued with additional biomolecules as part of its continuous exposure to circulating tissue fluids deriving from plasma (Triffitt *et al.* 1976).

Structure of craniofacial bone

Structural hierarchy in the skeleton creates a highly organized, robust mineralized tissue that performs a variety

of functions based on the biomechanical demands placed upon it at specific anatomical sites. In the craniofacial complex, these demands are largely protective (the cranium) and masticatory (moving jaws and their teeth). Craniofacial bones, not unlike bones found elsewhere, have a hierarchical structure reflecting, at least initially, either their intramembranous or endochondral origin, both types of which exist in the craniofacial complex. However, at the ultrastructural level, there appears to be little to distinguish them from bone found at other anatomical sites. At the macro scale, craniofacial bone is organized much like that found throughout the skeleton, with regions of dense cortical bone and regions of porous trabecular bone. However, intermediate structural organization is common, reflecting a continuum between dense and porous architecture. Regulation of bone growth patterns and rates within the craniofacial complex occurs through a large number of endocrine factors, including a significant influence of growth hormone (Pirinen 1995).

Trabecular bone spans marrow cavities found within dense cortical lamellar bone, and this occurs in the head as it does for long bones of the limbs as well as for the axial skeleton. For the craniofacial complex, this is evident in the diploe of the calvariae and in the tooth-supporting alveolar bone of the maxilla and mandible (Sodek & McKee 2000). This arrangement in the periodontium provides the structural basis for fixation and suspension of teeth within osseous alveolar sockets via the periodontal ligament inserting (as Sharpey’s fibers) into bone and tooth. Insertion sites of the periodontal ligament, both within dense alveolar bone and in tooth cementum (McCulloch *et al.* 2000), provide an

anatomical arrangement essential for masticatory function while simultaneously providing mechanosensory feedback during mastication.

At the more microscopic scale (Figure 12.1), bone structure continuously changes under the influence of

the cells that shape and remodel it, and even the mineral itself matures in such a way that early mineral is different from late (aged) mineral within a given volume of bone over time. Central to all bone formation processes in the craniofacial complex and elsewhere in the skeleton is

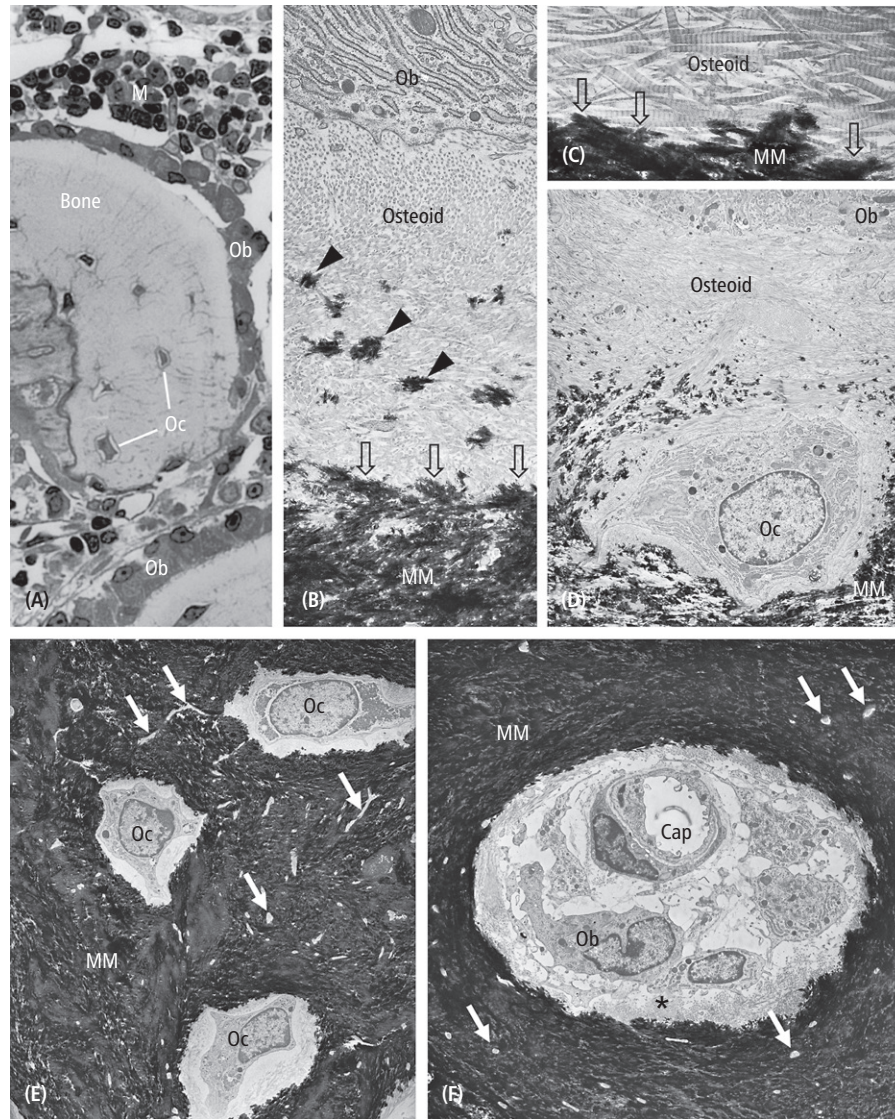


Figure 12.1 Light (A) and transmission electron (B–F) micrographs showing osteoblast-lineage bone cells, unmineralized bone extracellular matrix (osteoid), and the mineralized matrix (MM) of bone. A. Cuboidal osteoblasts align at forming bone surfaces with some becoming incorporated into the bone as osteocytes. B–C. Osteoblasts secrete a collagen fibril-rich layer of extracellular matrix known as the *osteoid* within which small mineralization foci (arrowheads) are present. Mineralization becomes more confluent at the mineralization front (open arrows) where it permeates throughout the fibrillar and interfibrillar matrix compartments. D–E. A proportion of osteoblasts becomes encased within extracellular matrix and is embedded within the bone as osteocytes. First known as *osteoid osteocytes*, they eventually become surrounded by mineralized matrix whose walls form the osteocyte lacunae, from which osteocyte cell processes extend into the mineralized bone matrix within small channels termed *canaliculi* (arrows). (F) In regions of compact bone, vascular channels with a central capillary (Cap) can be found surrounded by late-stage osteoblasts (soon to become bone-lining cells) adjacent to a minimal-thickness intervening osteoid (asterisk) which will disappear as a distinct layer as mineralization proceeds up to the bone surface (canaliculi, arrows). All images are taken of a one-month-old, glutaraldehyde-fixed rat palatine bone embedded in Epon epoxy resin and conventionally stained with toluidine blue (A) or uranyl acetate and lead citrate (B–F). Ob: osteoblasts; Oc: osteocytes; and M: marrow.

continual cell activity resulting initially in extracellular matrix deposition and assembly by osteoblasts, followed by its maturation by osteoblasts and osteocytes. As part of this process, extracellular matrix mineralization hardens the bone. Not only cell dynamics form the bone developmentally but also bone cell-signaling events provide the mechanism by which bone is constantly remodeled by osteoclasts (Bruzaniti & Baron 2006). Bone remodeling is either stochastic, to prevent it from exceeding a predetermined age, or targeted, to replace bone based on its use and the resulting biomechanical forces placed upon it (Parfitt 2002).

The extracellular matrix of bone mineralizes as part of all developmental processes leading to mature skeletal element formation (Karsenty & Wagner 2002), whether they occur in the craniofacial mineralized tissues or elsewhere. For bone turnover, mineralization is likewise a major physiologic and structural requirement that occurs by either stochastic or targeted bone remodeling. Conversely, dissolution of mineral occurs as a result of the pH-lowering activities of osteoclasts during bone resorption, and low pH is also required for extracellular matrix degradation by proteolytic enzymes that function optimally at acidic pH levels (Blair 1998). Bone-lining cells strategically situated at bone surfaces relay incoming signals from the osteocyte network regarding continuously changing stress-strain fields within the interior regions of the bone (Klein-Nulend *et al.* 2003). The mechanosensory osteocyte network is always present in bone wherever it is formed, but it is particularly active in regions of alveolar bone in the craniofacial complex affected by the transmission of frequent cycles of periodontal ligament tension between the teeth and the surrounding alveolar bone. In these regions of craniofacial bone, bone metabolism is particularly high, and extensive bone remodeling occurs as evidenced by the presence of abundant cement lines outlining reversal sites where bone deposition by osteoblasts was preceded by bone resorption by osteoclasts (McKee & Nanci 1995a). These reversal sites reflect the high bone turnover occurring in response to various signals derived from the repetitive biomechanical demands imparted by mastication, and likely represent an important adhesive interfacial boundary between older remodeled bone and newly deposited bone. More internally, deeper within compact dense cortical bone and visible at the microscopic level, osteonal bone remodeling creates roughly cylindrical Haversian systems (more irregular in craniofacial bones than in long appendicular bones, the latter being typically aligned with the long axis of the bones) that impart an additional level of strengthening architectural hierarchy. Hemi-osteonal remodeling occurs at the surface of trabecular bone and at the inner and outer surfaces of

cortical bone to ensure turnover and replacement of bone at these surface sites (Parfitt 2002). Altogether, these microscale changes created locally by cell-signaling molecules, and more distally by release and systemic circulation of hormones, ultimately result in a layered macro and micro (lamellar) bone structure whose spatial configuration meets local biomechanical demands. The hierarchical structure of these various elements in bone, like in other biologic and synthetic composite materials, prevents catastrophic material failure by creating interfaces that deflect microcracks to resist crack propagation leading to fracture (O'Brien *et al.* 2005).

Transcriptional regulation of osteoblast differentiation

The synthesis and deposition of bone matrix and its subsequent mineralization—to form the hierarchical bone architecture, as described in this chapter—are actively regulated by osteoblasts. Our current understanding of the regulation of the molecular events that regulate osteoblastogenesis is largely based on findings obtained from gene-targeted mouse models and from patients with congenital skeletal defects. Over the past 20 years, advances in gene manipulation techniques have made the mouse a very attractive model organism to study skeletal and dental biology since the phenotypic consequences of gene mutations that affect skeletal and dental development and functions are largely the same in mouse and human.

Osteoblast progenitors differentiate from pluripotent mesenchymal stem cells. Although these progenitor cells in the craniofacial, axial, and limb bones originate from different embryonic lineages—the neural crest, paraxial, and lateral plate mesoderm, respectively—their terminal differentiation involves the same signaling events and downstream transcription factors (Olsen *et al.* 2000).

Three key transcription factors regulate the osteogenic commitment of pluripotent mesenchymal stem cells and their terminal differentiation into functional osteoblasts. Runt-related transcription factor 2 (Runx2) is an early requirement for the differentiation of chondro- or osteogenic bi-potential progenitor cells (Ducy *et al.* 1997; Komori *et al.* 1997; Mundlos *et al.* 1997; Otto *et al.* 1997). In Runx2-null mice, osteoblast differentiation is arrested in both intramembranous and endochondral skeletal elements, resulting in a complete absence of bone tissue (Komori *et al.* 1997). The loss of one allele of Runx2 causes cleidocranial dysplasia (CCD) in both mice and humans (Mundlos *et al.* 1997; Otto *et al.* 1997). This autosomal dominant disorder has as its hallmark characteristics a delay in the closure of cranial sutures, hypoplastic or aplastic clavicles, and dental abnormalities

including a delay in tooth eruption. In mature osteoblasts, Runx2 regulates the expression of many secreted proteins, including osteocalcin and extracellular matrix proteins like those of the SIBLING (small, integrin-binding ligand N-linked glycoproteins) protein family (Ducy *et al.* 1997).

Once committed as chondro- or osteogenic bi-potential progenitors, differentiation into functional osteoblasts requires the expression of osterix (OSX), a transcription factor belonging to the specificity protein (SP) family (Nakashima *et al.* 2002). OSX acts downstream of Runx2, with its expression being specific to osteoblasts. In *Osx*-null mice, Runx2 expression and chondrogenesis are unaffected, but because of the lack of functional osteoblasts, no intramembranous or endochondral bones are formed (Nakashima *et al.* 2002).

Maintenance of osteoblast function (e.g., continuing extracellular matrix synthesis) requires activating transcription factor 4 (ATF4; Yang *et al.* 2004). ATF4 belongs to a family of widely expressed transcription factors commonly known as cAMP response element-binding proteins (CREB proteins). Together with Runx2, ATF4 promotes the expression of osteocalcin and other matrix proteins including posttranscriptionally regulating the synthesis of type I collagen—the most abundant protein in the bone matrix. Accordingly, ATF4-deficiency in mice leads to a delay in skeletal development and a low-bone-mass phenotype throughout the postnatal period (Yang *et al.* 2004).

Early events in bone extracellular matrix deposition

FN matrix in bone

Although type I collagen fibrils constitute the majority of the bone matrix (by weight), pre-osteoblasts (like most cells of mesenchymal origin) initially establish a provisional fibronectin (FN) matrix. FN is a large glycoprotein that can assemble under cellular control—mediated by integrins and the Arg–Gly–Asp (RGD) sequence found in FN—into an insoluble fibrillar matrix. Subsequent to this, FN acts as a scaffolding network in bone and other connective tissues for the assembly and deposition of many other permanent matrix constituents that include thrombospondin, fibrillin-1, and collagen type I (COL I; Sottile & Hocking 2002; Mao & Schwarzbauer 2005). Provisional FN matrix can disassemble via unknown mechanisms under circumstances where tissue formation is not desired (Sottile & Hocking 2002), or it can be further modified to become permanent together with the process of COL I deposition. Bone FN derives from two sources: serum (as produced by liver hepatocytes) and resident osteoblast lineage

cells, the former being particularly important in maintaining bone matrix and bone mineral density, while the role of the latter is less well understood. Conditional deletion of Fn1 in liver hepatocytes in mice results in osteopenia indicating that circulating serum FN regulates bone mass. Circulating serum FN permeating into tissue fluids readily diffuses into both osteoid and mineralized bone matrix and indeed is the major source of the FN found in bone. Osteoblast lineage-derived FN regulates osteoblast function (mostly osteoblast numbers) in an RGD-independent manner, but it does not affect COL I matrix formation in bone as demonstrated in mice via conditional Fn1 deletion in osteoblasts (Bentmann *et al.* 2010). Additional *in vitro* studies have also demonstrated that the provisional FN matrix mostly derives from the circulating exogenous pool of FN. In support of this, Fn1-null fibroblasts assemble normal matrix when exogenous FN (the serum form) is supplied in the growth or culture medium (Sottile & Hocking 2002).

While the details and mechanisms underlying how provisional bone matrix becomes permanent are still largely unknown, it is plausible that the process involves matrix stabilization via protein cross-linking between FN and COL I via the actions of transglutaminase enzymes. Heterotypic cross-linking between these two matrix proteins has not yet been shown *in vivo*, but FN has been identified as a transglutaminase 2/Factor XIIIa substrate *in vitro* in osteoblasts (Al-Jallad *et al.* 2006). It is also possible that FN cross-linking could simply induce a conformational change in FN to increase its affinity for COL I or produce changes in FN structure that would increase its stability to block disassembly resulting in generation of the subsequent, more permanent FN-COL I matrix.

Matrix stiffness and osteoblast differentiation

Osteoblast differentiation, in addition to being promoted by myriad signaling pathways, is also controlled by the immediate matrix environment. Osteoblasts interact with many matrix proteins in their surrounding milieu and, in doing so, sense matrix stiffness. When plated *in vitro* onto a substance with the same surface chemistry but with different material stiffness, mesenchymal stem cells differentiate into either neural cells, muscle cells, or osteoblasts, with higher material stiffness favoring osteoblast differentiation (e.g., assessed by osteocalcin gene expression; Engler *et al.* 2006). Although very little information is available on the factors imparting stiffness to early bone matrix other than what is known about the rigidity of the collagen fibrils themselves, bone matrix stiffness most certainly is determined by the many homotypic and heterotypic interactions

occurring within the network of matrix proteins. Such interactions would likely include additional covalent protein cross-linking reactions to stabilize and render robust properties to bone extracellular matrix, while at the same time being permissive of mineralization.

Before mineralization, and at a stage of early matrix deposition, osteoblasts additionally produce matrix protein cross-linking enzymes such as lysyl oxidase and transglutaminases that likely determine initial matrix stiffness properties. Such extracellular modifications lead to “autocrine” stiffness sensing by osteoblast integrins whereby mechanical signals from such matrix cues are converted into intracellular biochemical-signaling cascades. Such a biomolecular process involving integrins and their ligands in osteoblast lineage cells would typically include integrin clustering into focal adhesions that activate downstream signaling pathways seen for many cells (e.g., FAK, RhoA GTPase, ROCK and/or MAP kinase pathways), which, in turn, modulate gene expression patterns for osteoblast differentiation and function (Shekaran & Garcia 2011).

Extracellular matrix assembly, composition, and maturation

The sum total of all protein–protein/glycan interactions, together with the properties of the apatitic mineral phase itself and the extensive surface area of the organic-inorganic interface where matrix and other organic molecules interact with the mineral crystal surfaces, provide bone with its unique material and biomechanical properties. Clearly, as described above, the fundamental organic scaffolding element is type I collagen fibrils, but when considered not on a weight or volume basis (where it comprises 85% to 90% total bone matrix protein by weight) but rather, on a molar basis, total noncollagenous proteins, small proteoglycans, and other organic moieties constitute roughly similar amounts of organic matrix as does collagen (Robey 1996). While only recently becoming an extensive area of study (as compared to type I collagen, which has been the subject of analysis for decades), relatively less is known about the roles, structures, and networks of the noncollagenous proteins and proteoglycans. This prominent “pool” of noncollagenous matrix molecules in bone has long been thought to be of significant importance in providing a mineralization-competent environment for the growth and regulation of the apatitic crystals that uniquely define bone and teeth (Kawasaki & Weiss 2006). Craniofacial bones generally appear to have a matrix composition and structure not unlike those of skeletal bones elsewhere. During development, the more interwoven (loosely packed and randomly oriented collagen fibrils)

nature of so-called woven bone formed early in skeletogenesis (particularly in utero) contains a higher percentage of noncollagenous proteins and glycans than does the denser, lamellar bone formed mostly postnatally where collagen fibrils are densely packed and aligned in alternating sheets (lamellae).

Collagen fibrils in bone are formed primarily from type I collagen molecules (>95%) with small amounts of type V (<5%) that can form heterotypic fibrils with type I collagen, and even smaller amounts of other collagens can be found (Veis 1975). The triple-helical structure of collagen derives from a repetitive Gly-X-Y sequence of amino acids in the central region of each alpha chain, with proline and hydroxyproline commonly residing at the X and Y positions, respectively. Collagen fibrils are 10–300 nm in diameter and can be many micrometers in length, and they have a characteristic banding pattern when examined by electron microscopy consisting of collagen molecules quarter-staggered into a 64–67 nm periodic array of hole and gap zones. The high tensile strength characteristic of fibrillar collagens is attributable to covalent cross-links between lysines and modified lysines within and between collagen molecules, occurring as a result of lysyl oxidase enzymatic activity (Eyre & Oguchi 1980). The cross-links are diverse and complex and occur at different regions within and between the collagen molecules, and maturation of the cross-links can occur through subsequent interactions with additional lysine-derived side chains to form trifunctional cross-links such as pyridinoline (consider by some as a diagnostic marker of bone resorption; Eyre *et al.* 2008).

Whereas details of collagen fibrillogenesis in all connective tissues including bone are well known, relatively little is known about how noncollagenous protein networks are established, and how they might function in concert with the collagen network (details on individual proteins are given in the “Mineralization of Bone” section). Networks of FN established early in matrix deposition (discussed in this chapter) are perhaps the best characterized among the noncollagenous proteins, and these are clearly very important to establishing the subsequent, more permanent, collagen network. Many noncollagenous proteins in bone are substrates for the cross-linking activity of the transglutaminase enzymes expressed by osteoblasts, and these enzymes form homotypic and heterotypic complexes of various noncollagenous proteins that can be found in bone (Kaartinen *et al.* 2002), but their functions are unknown. Such transglutaminase activity on this class of matrix proteins needs further study but likely represents a functional counterpart to collagen cross-linking, where the former is important for cell signaling and the regulation of

mineralization in bone, with the latter providing a robust scaffolding important for the mechanical properties of bone.

Small, leucine-rich proteoglycans (SLRPs) such as decorin and biglycan are found in bone (Young *et al.* 2006) and consist of a protein core and one (decorin) or two (biglycan) glycosaminoglycan side chains of chondroitin-4-sulfate. In bone, the SLRPs may account for up to 10% of the noncollagenous proteins, but they decrease with age. The domain structures of decorin and biglycan indicate that these two proteoglycans likely do not act alone, but bind to other matrix components to exert their function (Roughley & Lee 1994).

Finally, mentioned only briefly in this chapter were categories of the most prominent proteins found in bone; there are many others. In addition to the local cellular production of secreted organic constituents, bone is a repository for many circulating plasma and tissue fluid proteins, heavy metal ions, and dietary and toxic substances.

Mineralization of bone

The extracellular matrix produced by osteoblasts ultimately becomes fully impregnated with mineral, both intra- and interfibrillarly (with respect to collagen fibrils) forming a nanocomposite organic-inorganic hybrid material. Once the mineralization-competent extracellular matrix is established by osteoblasts, at certain punctate locations within the osteoid or at a deeper plane within the osteoid called the *mineralization front*, calcium-phosphate mineral deposition commences (McKee *et al.* 2005). This mineral deposition in intimate association with organic material is not unique to vertebrates, as biominerals are found everywhere throughout the animal and plant kingdoms where they cut across most phyla. Surprisingly, or perhaps not surprisingly to some, the chemical composition and atomic structure of macroscopic and microscopic rocks found in the crust of the earth and in the geologic displays of museums and gem collectors are essentially the same as those used in vertebrate biology to harden craniofacial tissues and other skeletal elements. The difference, of course, lies in the fact that biology has spatially diminished this rock-forming process down to the nanoscale to form “nanorocks” in bones and teeth, all carefully controlled and guided by extracellular matrices and other biomolecular determinants. Where biomineralization occurs within extracellular matrices, as in vertebrate bone, cartilage, dentin, cementum, and enamel, the end result is billions (if not trillions) of nanocrystallites embedded within an organic scaffolding that together afford a degree of tissue flexibility and toughness. Gene mutations

that ultimately affect mineralization-regulating proteins (including enzymes) typically lead to bone and tooth nanocrystallites defective in number, size, shape, and/or orientation and can potentially lead to changes in mineral type such that these tissues become diseased, soft, and/or brittle.

During mineralization of the skeleton (and the dentition except for enamel), heterogeneous mineral deposition events occur at discrete locations within, at the surface of, and between collagen fibrils (Landis *et al.* 1996; McKee *et al.* 2005). This mineralization defines the composite nature of bone that spans most dimensional scales starting from the protein/organic–nanocrystal interface and extending to the cement line and beyond into the anatomical macroscale structure of bones. The collagen fibrils, the noncollagenous protein assemblies, the interconnected pores within the structured extracellular matrix, and the apatitic crystals of bone all have nanoscale dimensions. Mineral is nucleated not only in nanoscale volumes within fibrillar collagens but also interfibrillarly where crystal dimensions are slightly larger and where noncollagenous proteins are abundant (McKee & Nanci 1995b). These two structurally and biochemically distinct extracellular compartments provide different constraints on biomineralization, yet despite this, mineral is similarly nucleated at each site, and crystal growth is subsequently regulated such that bone crystals do not grow beyond the nanometer range. Initially, to reach an apatitic mineral phase, critical nuclei of mineral ions reach a size and stability that subsequently mature to form crystals having some degree of long-range order that ultimately possess well-defined and crystallographic faces characteristic of a substituted hydroxyapatite mineral phase. Initial nucleation events are controlled locally by concentrations of mineral ions and by the presence, or absence, of mineral nucleators and inhibitors (Boskey 2003). For bones and teeth, the crystals typically are platelet-shaped crystallites with dimensions that vary depending on their physical location within the structure of the pre-established extracellular matrix (Landis *et al.* 1996) with different local chemical environments afforded by matrix composition defining crystal properties at those sites. Charge groups and matrix interaction domains in fibrillar type I collagen have been characterized, but much less is known about the chemical environment contributing to mineral crystal nucleation and propagation outside the fibrils. While crystal deposition within the collagen fibril is thought to occur independently from that between the fibrils, Toroian and colleagues (2007) have recently proposed a linked mineralization process in which crystals formed at the surface of the fibrils might migrate to locations within the fibril.

Regardless of the location within the extracellular matrix, the exquisite biological control over bone mineralization is thought to be regulated by mineral-nucleating and mineral-inhibiting proteins (and their released peptides; George and Veis 2008) or by the local removal of inhibitors (such as pyrophosphate by tissue-nonspecific alkaline phosphatase) from the extracellular matrix (discussed in this chapter). Many of the noncollagenous proteins in bone (and in teeth, and many other biomineralized invertebrate tissues) are acidic phosphoproteins that bind strongly to mineral, often through negatively charged phosphate groups, to regulate crystal growth. Evidence for the role of acidic, negatively charged mineral-binding proteins (and their peptides) being important in regulating at least the interfibrillar mineralization process is accumulating; this family of proteins (which also has cell-signaling properties), referred to as the SIBLING family (Fisher & Fedarko 2003), exists as a subcategory of the secreted calcium-binding phosphoprotein (SCPP) family (Kawasaki & Weiss 2006). Abundant evidence exists to show that in various mineralized tissues (including pathologically mineralizing soft tissues), crystal growth, once initiated, is regulated (inhibited) by direct binding of inhibitory proteins or peptides (e.g., osteopontin and its ASARM-containing peptides) to crystal surfaces (Addison *et al.* 2010; Giachelli & Steitz 2000).

While a reasonably large body of information exists on how crystal growth might be regulated once mineral has formed, relatively little information is available on the earliest steps in the mineral induction process. However, recent progress centered around the tissue-nonspecific form (versus intestinal and placental forms) of the enzyme family of alkaline phosphatases has provided exciting new insight into the role of this enzyme, which is produced at high levels in mineralized tissues. Tissue-nonspecific alkaline phosphatase (TNAP; also known as TNSALP, ALPL, AKP2, and liver, kidney, or bone alkaline phosphatase), a well-known early osteoblast marker, is capable of removing phosphate groups from extracellular matrix phosphoproteins and by doing so can modify their mineral-binding (inhibitory) capacity and potentially provide a source of phosphate for mineralization (Addison *et al.* 2007). A thorough review of the alkaline phosphatase family of enzymes has been provided by Millan (2006).

The most prominent enzymatic function of TNAP in mineralized tissues was postulated many years ago by Fleisch and colleagues (1966) who suggested that its function might be related to degradation of mineralization-inhibiting pyrophosphate (P_2O_7), a byproduct of many metabolic intracellular processes. Indeed, it is a potent inhibitor of bone (and tooth dentin and cementum)

mineralization (Harmey *et al.* 2004; McKee *et al.* 2011). Bisphosphonates, widely used clinically to treat osteoporosis, Paget's disease, and osteogenesis imperfecta, are pyrophosphate analogues having a nonhydrolyzable carbon atom replacing the central oxygen, with intentionally modified variable side groups conferring different cytotoxic potencies that are used principally to affect bone-resorbing osteoclasts by being toxic to various intracellular metabolic pathways upon uptake by these cells.

The membrane protein ANK (a progressive ankylosis gene [Ank] encoding a multiple-pass transmembrane protein, ANK) transports pyrophosphate across the cell membrane into the extracellular environment or, alternatively, pyrophosphate can be produced outside the cell directly from extracellular nucleotides through the action of the membrane protein ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1, also known as NPP1, NPPS, and PC1; Harmey *et al.* 2004). Studies in transgenic mice lacking these membrane proteins and thus not providing adequate extracellular inhibitory pyrophosphate levels show increased extracellular calcification, whereas mice that are deficient in TNAP conversely show decreased extracellular mineralization because of elevated amounts of inhibitory pyrophosphate present extracellularly in the absence of this enzyme (for review, see Murshed & McKee 2010). Human hypophosphatasia results from loss-of-function mutations in the TNAP (ALPL) gene, and in the skeleton and dentition, elevated pyrophosphate in these patients leads to hypomineralized bone and tooth extracellular matrices. Of clinical note, transgenic mice that are deficient in TNAP and have hypomineralized bones and teeth, when treated with enzyme replacement therapy using a mineral-targeting form of TNAP, show a fully mineralized skeleton and dentition (Millan *et al.* 2008; McKee *et al.* 2011).

The enzymatic action of TNAP on pyrophosphate cleaves this inhibitor to produce two phosphate ions, and by doing so, simultaneously removes a small-molecule inhibitor of mineralization and generates two phosphates potentially adding to the pool of phosphate ions available for apatitic crystal growth. Of these functions, it is generally believed that the removal of pyrophosphate as an inhibitor is more dominant in facilitating mineralization. A schematic summary of the major players thought to be involved in the induction of mineralization is presented in Figure 12.2; some of these determinants may also pertain to matrix vesicle-induced mineralization (discussed in this chapter).

Alternatively, or as an additional mechanism initiating mineralization, membrane-bounded matrix vesicles released by chondrocytes, osteoblasts, odontoblasts, and

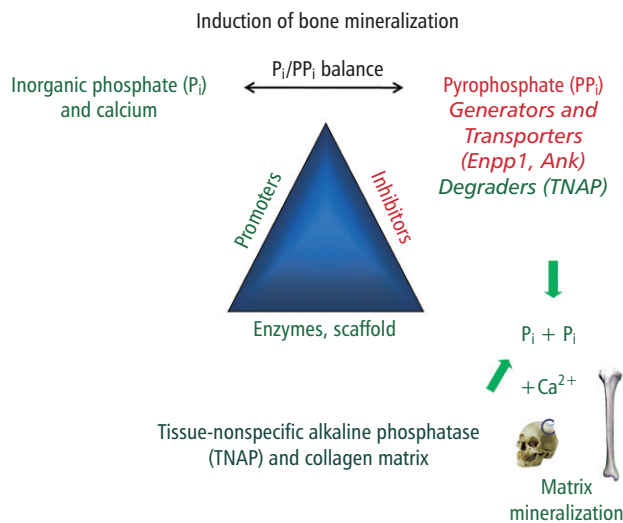


Figure 12.2 Induction of bone mineralization. Summary diagram illustrating the relationships among mineral ions and promoters and inhibitors of mineralization for inducing mineralization in bone, and additional requirements for a fibrillar matrix scaffolding and for enzymes to remove mineralization inhibitors, as described in this chapter. In particular, recent work has shown the importance of shifting the phosphate–pyrophosphate (P_i – PP_i) balance as a central control mechanism for inducing or inhibiting mineralization in bone such that decreases in inhibitory PP_i induce mineralization.

cementoblasts have also been proposed to initiate mineralization at specific sites within the extracellular matrix (Anderson 2003). In this latter case, crystals form within the vesicle under the control of vesicular components including integral proteins of the limiting membrane. The earliest events in matrix vesicle mineralization appear to involve intravesicular calcium and phosphate influx and PHOSPHO1 enzymatic activity. PHOSPHO1 is a phosphatase with specificity for phosphocholine and phosphoethanolamine whose activity increases intravesicular phosphate content by releasing phosphate from membrane phospholipid derivatives to shift the phosphate–pyrophosphate balance in favor of mineral deposition (Roberts *et al.* 2007). Following initial crystal growth within the matrix vesicle and rupture of the vesicle membrane by extended crystal growth, extracellular matrix molecules that otherwise regulate nonvesicle-mediated matrix mineralization (as described in this chapter) would proceed in their capacities to control crystal growth (Yadav *et al.* 2011).

Mineralization in the skeleton is also controlled systemically by the release of many circulating factors ultimately functioning to modulate serum concentrations (and thus tissue fluid concentrations in bone) of calcium and phosphate. Clearly, a requirement for physiologic

levels of these important mineral ions provides the basis for normal crystal growth in bone, cartilage, and teeth. When mineral ion homeostasis is sufficiently perturbed to result in subthreshold concentrations of circulating calcium and phosphate, rickets or osteomalacia (hypomineralization) develops, resulting in soft skeletons showing fractures and bone deformities. In 2008, Quarles reviewed the role of circulating factors in regulating mineral ion homeostasis, while here we have focused on factors directly regulating crystal growth within the extracellular matrix of bone.

Cell–matrix and matrix–matrix interfaces in bone

Cell–matrix interfaces

Interfaces in bone can be broadly classified as being either cell–matrix or matrix–matrix interfaces. The former category predominantly comprises the interaction of the various bone cell lineages with surfaces of bone, and the latter sites where newer bone is deposited onto older bone (or also onto calcified cartilage in the case of endochondral ossification) to create an interface between two spatially and temporally distinct matrices (see the “Matrix–Matrix Interfaces” section). During essentially all stages of both bone formation by osteoblast-lineage cells and bone resorption by osteoclasts, important extracellular information is detected by cells, and as a result, signals are transmitted across the cells that affect gene expression and cellular activity. Indeed, bone formation and turnover appear to be tightly regulated by a multitude of local and systemic cell-signaling mechanisms, many of which seem to reside in the way in which bone cells respond to stimuli generated by the solid-phase substrates with which they come into contact. In bone, whose primary weight-bearing and protective roles are defined by its rigid, collagenous, and mineral-impregnated extracellular matrix, exposed bone surfaces are abundant, yet these are populated by a relatively small set of bone cells that includes only four differentiated cell types: osteoblasts, osteocytes, bone-lining cells, and osteoclasts. Each of these cells interacts in a well-defined way with adjacent matrix (inside-out and outside-in signaling) to ensure that the various metabolic processes occurring in bone do not occur haphazardly but rather proceed in a coordinated, appropriate manner that is capable of responding to the biomechanical demands, and homeostatic activity, intrinsic to this tissue.

Cell–matrix interfaces are usually characterized by the presence of a variably thin lamina limitans measuring only nanometers in thickness and thus requiring visualization by transmission electron microscopy. The lamina

limitans is a protein film—rich in osteopontin—found at bone surfaces interfacing with cells; these interfaces include the periphery of osteocyte lacunae and bone canaliculi, quiescent bone surfaces covered by bone-lining cells, and bone surfaces covered by osteoclasts (McKee & Nanci 1996). Given its cell adhesion tripeptide RGD sequence and other cryptic integrin-binding motifs, together with its mineral-binding properties, osteopontin (hence its original naming referring to *bone bridging*) is thus well positioned in the lamina limitans to mediate cell matrix binding and signaling. Other SIBLING proteins in the matrix, such as dentin matrix protein 1, might play similar roles. Integrin-binding proteins located at bone surfaces, when ligated, would be expected to function in sensing bone strain and fluid-flow motions as occurs in mechanosensing in bone mediated by osteocyte, osteoblast, and bone-lining cell processes in the lacuno-canalicular network. Likewise, similar proteins concentrated at bone surfaces destined for resorption by osteoclasts presumably facilitate initial osteoclast attachment and migration, processes shown to be modified by the phosphorylation state of surface-bound osteopontin, with dephosphorylation being achieved enzymatically by the release of tartrate-resistant acid phosphatase (TRAP) by osteoclasts (Ek-Rylander & Andersson 2010).

Matrix–matrix interfaces

Mineralization processes occur *de novo* and on an ongoing basis in bone formation; they also occur related to the bone remodeling cycle at a time where osteoclastic resorption has finished at a particular remodeling site. Resorption of bone by osteoclasts creates a new tissue interface where an osteopontin-rich cement line (really cement plane, when considered in three dimensions) is subsequently deposited by osteoblast-lineage cells immediately prior to their differentiation into more mature, collagen-producing osteoblasts (McKee & Nanci 1996). Thus, secretion of osteopontin seems to be one of the earliest matrix secretion events by (pre)osteoblasts during the bone remodeling cycle. Secreted osteopontin accumulates against the resorbed bone surface, potentially acting as an adhesion promoter between the older bone and the newer bone, “priming” that surface for subsequent cell adhesion and/or cell-signaling events, or regulating early stages of the mineralization process as the cement line mineralizes to bond newer bone to older bone. Consistent with these roles for osteopontin is that it is a substrate for the covalent cross-linking activity of transglutaminase 2 (Kaartinen *et al.* 2002), that it contains several integrin-binding motifs (Lund *et al.* 2009), and that it is a potent regulator of mineralization (Addison *et al.* 2007). An extension of understanding the

cycle of bone formation, mineralization and remodeling is the notion that differentiating osteoblasts might behave similarly when confronted with the surface of an implanted material or after encountering a surgically created or fractured osseous interface (McKee & Nanci 1996)—all being surgical or trauma events frequently applied to, or occurring in, craniofacial bone. Interfacial recognition (or nonrecognition) of a solid-state substrate likely would provide a similar cascade of signaling events to ensure osseointegration or new bone fill at a defect or fracture site.

Medical and dental health implications

This chapter has covered fundamental aspects of bone cell differentiation, extracellular matrix production, and mineralization. The skeleton, and importantly the bones (and teeth) of the craniofacial complex, are normally constructed in such a way as to be as robust as possible for their supportive, protective, and masticatory functions. Apart from the obvious requirement for sufficient numbers of bone cells to do their respective tasks in healthy bone, the composition and quality of the organic extracellular matrix and the inorganic mineral phase contained therein are key determinants of healthy bones and teeth. Defects at almost any level of the extracellular matrix in mineralized tissues typically result in soft and/or brittle bones and teeth, with well-known examples being osteogenesis or dentinogenesis imperfecta (collagen gene mutations) and autosomal recessive hypophosphatemic rickets (dentin matrix protein 1 gene mutations). Likewise, inactivating mutations in several genes encoding for enzymes important in facilitating mineralization also result in soft, hypomineralized (osteomalacic) bones prone to deformities and fracture, with well-known examples being X-linked hypophosphatemia (phosphate-regulating endopeptidase homolog, X-linked [PHEX] gene mutations) and hypophosphatasia (tissue-nonspecific alkaline phosphatase [TNAP, ALPL] gene mutations). Odontohypophosphatasia (and some other forms of hypophosphatasia) resulting from ALPL mutations results in premature tooth loss caused by an absence of mineralized acellular cementum at the tooth root surface.

Summary

Craniofacial bones have a structure, composition, and mineralization pattern not unlike bones seen elsewhere in the axial and appendicular skeleton. Structural hierarchy in the skeleton creates a highly organized, robust mineralized tissue that performs a variety of functions based on the biomechanical demands placed upon it at specific anatomical sites. In the craniofacial complex,

these demands are largely protective (the cranium) and masticatory (moving jaws and their teeth), and the hierarchical structure of these skeletal elements reflects, at least initially, either their intramembranous or endochondral origin—both types of which exist in the craniofacial complex. Osteoblast-produced extracellular matrix, rich in collagen fibrils and many other noncollagenous proteins and small proteoglycans, provides the scaffolding network for calcium–phosphate (apatite) mineralization. Within this extracellular matrix, the sum total of all protein–protein/glycan interactions, together with the properties of the apatitic mineral phase itself and the extensive surface area of the organic–inorganic interface where matrix and other organic molecules interact with the mineral crystal surfaces, provide bone with its unique material and biomechanical properties.

Future directions and perspectives

Mineralization studies in vertebrate tissues are unique in that they reside at the intersection of biology and geology. Moreover, biomineralization in various organisms is as diverse as it is complex, involving myriad small-molecule, protein–enzyme, proteoglycan, and lipid derivatives as determinants of extracellular matrix, and intravesicular, mineralization. Some of these factors circulate systemically, while others reside and act locally, directly at sites of crystal growth. For mineralized tissues in the craniofacial complex of humans, which include bone and cartilage, tooth dentin, cementum and enamel (all with apatitic mineral), and inner ear otoconia (with calcitic mineral), much remains to be learned about how such exquisitely fine, nanoscale control is exerted over mineral crystal growth occurring within a veritable “soup” of biologically active agents. Molecular mechanisms for extracellular matrix assembly and crystal growth need to be understood at the level of the organic–inorganic interface residing within the extracellular matrix where mineralization occurs. The deciphering of these specific processes will require the combined, interdisciplinary skills and methods of both biologists and geologists alike, along with many others, to translate to the clinic new ways to detect, treat, and repair defective mineralized tissues in the craniofacial complex and elsewhere.

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Clinical correlate: osteogenesis imperfecta

Peter H. Byers

This is a work of fiction and no resemblance is intended to any person living or dead, with the exception of the idea of his presentation. Figure 13.1 provides a classical case study of dentinogenesis imperfecta, generously provided by Dr. Rebecca Slayton from the University of Iowa.

Case presentation

George B. is a 38-year-old man who recently settled in Seattle, Washington, USA, to take a new job in product development with Microsoft. He was born with a fractured femur that healed well; his second fracture occurred when he was approximately one year old and had just begun to walk. He was able to walk independently, but when he began to go to school, he was small and preferred to use a wheelchair because of concern about being knocked down and incurring additional fractures. He experienced approximately 30 fractures, continued to use a chair for mobility, but was independent in all skills of daily living. He drove independently. He graduated from college with a master's degree in engineering.

George's primary teeth were discolored, fractured, and worn down. His secondary teeth were also discolored, but the involvement varied among the teeth with relative preservation of the incisors and premolars. He wanted to preserve his dentition as long as possible to avoid the use of false teeth. On examination, he was a little more than 4 feet tall and had slightly gray sclerae, a palatal and slightly breathy voice with a high pitch, and a bifid uvula. His teeth were brown and gray; some were chipped, and some were worn almost to the gum.

George took no medications, had not been given bisphosphonates, and had been under good medical care. He was intelligent, knew a good deal about the basics of osteogenesis imperfecta (OI), had a dominant

mutation in a type I collagen gene that accounted for his clinical picture, and had elected to adopt a child with his wife rather than go through the issues surrounding prenatal diagnosis.

Osteogenesis imperfect and dentinogenesis imperfecta: presentations and basic defects

That it is possible to make a diagnosis of the progressive-deforming variety of osteogenesis imperfecta (OI type III) by telephone simply by listening to the opening question "Is that you, doctor?" illustrates the power of a single nucleotide change to influence the shape and function of the face, the skull, and their internal architecture. Adults with this disorder often have a higher pitched voice than expected, with a tambour that reflects the structure of the face and sinuses—they give the voice a breathy, reedy, "palatal" quality that is unique to this group of individuals. The same molecular defect that leads to the characteristic voice produces an instantly recognizable (for the experienced clinician) set of facial features—a broad forehead, pointed chin and triangular face, protuberant jaw, and malocclusion with the mandible forward of the maxilla—in addition to short stature and long-bone deformity. It is nothing short of astonishing to recognize that single nucleotide substitutions in one copy of one of the two genes, *COL1A1* and *COL1A2*, that encode the chains of type I collagen can result in this clinical picture (Marini *et al.* 2007).

Collagens: the model of structural proteins

Collagens are extracellular matrix proteins characterized by the presence of a repeating Gly-X-Y peptide sequence in which X and Y are amino acids other than glycine and are often proline and 4-hydroxyproline in those



Figure 13.1 A. Frontal view showing anterior open bite, excessive wear of mandibular incisors, and amber, opalescent teeth typical of dentinogenesis imperfecta. B. Maxillary occlusal view showing opalescent teeth, significant wear on molars, and narrow, v-shaped palate. Triage included temporary restorations placed in the second primary molars under general anesthesia prior to treatment. C. Mandibular occlusal view showing opalescent teeth with extreme wear of the incisors. D. Maxillary periapical radiographs showing large pulp chambers and abscessed maxillary left second molar.

respective positions, and from which tryptophan and cysteine are excluded. This gene family comprises some 40-plus members, each of which encodes a protein that adheres to the rules of sequence and formation of a trimeric structure with three chains. In some cases (type II collagen—encoded by the *COL2A1* gene and type III collagen—encoded by the *COL3A1* gene), these are homotrimers, while in other cases, type I collagen being the archetypical example, they are heterotrimers composed of the products of two or three genes. Type I collagen is encoded by two genes, *COL1A1* and *COL1A2*, with the pro α chain products of these genes, pro α 1(I) and pro α 2(I), respectively, incorporated in a 2:1 ratio into trimers.

The complex pathway of biosynthetic processing of collagens includes (Myllyharju & Kivirikko 2004):

- Splicing of the mRNA, and transport of the mature mRNA to the cytoplasm
- Initiation of translation
- Binding of the ribosome to the rough endoplasmic reticulum
- Insertion of the elongating chain into the lumen of the rough endoplasmic reticulum
- 4-hydroxylation of prolyl residues in the Y-position of the triple helical domain
- Hydroxylation of some lysyl residues in Y-position
- Regulated association of chains into trimers
- Folding of the chains into the requisite structure

- Secretion through the Golgi and a complex secretory system
- Cleavage of amino-terminal and carboxyl-terminal propeptides in the extracellular space
- Fibril formation

It is evident from this list that the margin for error is small and that the potential for mutations that affect both the primary structure and processing is large. Although about 90% of people with OI have mutations in the two type I collagen genes, more than eight additional genes have been implicated in mutations that result in the rare recessively inherited forms of OI. Most of these genes affect processes that range from chain association to postranslational modification and cross-link formation.

Proteins of the matrix and mutations and their consequences

The formation of the disparate parts of the craniofacial structures represents the endpoint of a finely orchestrated set of interactions among elements in the genome that include master regulatory genes, specific gene transcription factors, regulatory RNA sequences, transcription factors, splicing factors and regulatory elements, the translational machinery, the secretory pathways, and protein interactions at most points along the way. In the end, the diversity of human appearance that allows us to

distinguish one individual from another, yet sometimes confuse one person with another, is driven by the enormous allelic variation in our genomes. We divide the effects of this variation into two sets—one that we recognize to be “normal” and another that we define as out of the normal range and that we designate with syndromic names, for example *osteogenesis imperfecta*, *Marfan syndrome*, *Stickler syndrome*, and *achondroplasia*, to name a few. This distinction provides us with the substrate to identify single genes that can have significant effects on the formation of craniofacial structures and, we hope, will help to target processes or molecules that we can alter to effect a significant clinical benefit. The process requires that we identify the genes that harbor mutations, understand how those alterations change protein interactions, and then target processes that will restore development or function to a neutral state. All in all, this is a tall order and one in which we have not succeeded for most disorders. The examples that follow provide the current status for mutations that affect different elements in the matrix and outline the ways that therapies have been developed and their success or limitation.

Mutations that alter either the primary sequence of the proteins or the sequence and function of the modifying proteins (some are enzymes like lysyl hydroxylases and prolyl hydroxylases that modify the proteins, and others are proteins that assist in the folding of the proteins and the final quality control that assures that each part of the final molecule is picture perfect) can all result in recognizable clinical alterations. Different mutations in the type I collagen genes result in a spectrum of phenotypes. Most result in different types of osteogenesis imperfecta (OI) in which the presentations range from death in the perinatal period to a small increase in the lifetime incidence of fracture (Byers & Cole 2002). This reflects the presence of type I collagen as the major protein in bone. In the more severe forms of OI, accompanied by short stature, dentinogenesis imperfecta (DI) is a frequent co-traveler, which reflects the high content of type I collagen in dentin. A similar change in teeth, without the skeletal fragility and deformity, occurs with mutations in dentin sialophosphoprotein (DSPP). The primary teeth are generally discolored—usually gray or brown—and are brittle and soft. The teeth may fracture and wear easily. The secondary teeth are similar although they may be more robust. Frequently, however, they are ground down by usual daily processes and require attention. Use of protective coatings, and in some instances, implants may be helpful although the quality of mandibular and maxillary bone may require altered technical approaches. Most individuals with OI have dominant mutations in the type I collagen genes (isolated DI is also a dominantly inherited condition), which means that only one of the two copies of either gene is altered.

Usually those individuals with no family history of the condition develop the condition as a result of a new mutation that occurred in the process of egg or sperm formation and can transmit OI to their offspring—a 50% chance of transmission with each pregnancy.

Molecular mechanisms of genetic disorders

Mutations in the genes that encode the processing proteins generally result in recessively inherited disorders (it takes a mutation in each of the two copies, one inherited from each parent), many of which are also forms of OI. Like mutations in the type I collagen genes, mutations in these genes can produce clinical pictures in which bony alterations predominate and in which the severity ranges from lethal in the perinatal period to a demonstrable clinical involvement. DI is generally not part of the range of clinical expression of these mutations for reasons that are not clear, as the alterations seen in the type I procollagen molecules are often difficult to distinguish from those seen when the type I collagen genes are, themselves, the substrate for mutation.

Mutations in the genes that encode other modifying proteins, like the lysyl hydroxylation system, produce clinical pictures that vary widely, and depend on the specific gene involved and the targets for posttranslational modification. For example, *PLOD1* encodes lysyl hydroxylase 1, which has a specificity for the triple helical lysyl residues in type I procollagen. Mutations result in Ehlers–Danlos syndrome type VI, characterized by a Marfanoid habitus, intractable scoliosis, a risk for arterial aneurysm, dissection, a rupture, and an ocular globe rupture (Yeowell & Walker 2000). Mutations in a second gene, *PLOD2*, which encodes lysyl hydroxylase 2 that has a specificity for lysyl residues located just outside the triple helical domains and involved in cross-link formation in type I collagen in bone, result in a recessive form of OI with contractures, known as *Bruck syndrome* (Bank *et al.* 1999).

A third collagen gene, *COL2A1*, encodes the chains of type II collagen, the major collagen of cartilage and the vitreous of the eye. Like mutations in type I collagen genes, alterations in this gene can result in a wide range of clinical pictures that result from alterations in the structure or expression of one or both alleles. Because type II collagen plays a role both in the early modeling of the anlage of bones during development and in the formation of the growth plate, mutations in this gene can produce some of the most profound disorders of growth and early death, as in achondrogenesis type II (Körkkö *et al.* 2000), as well as subtle alterations that can result in early osteoarthritis, cleft palate or bifid uvula, and high myopia and retinal detachments in individuals with Stickler syndrome (Tiller *et al.* 1995).

Table 13.1 Osteogenesis imperfecta classification, clinical characteristics, mode of inheritance, and genes. As can be seen from this table, there are fundamentally two ways to classify OI: the clinical perspective and the genetic (causative gene) perspective. This table is in evolution, and the clinicians and geneticists in the field have not come to either grips or agreement with how to handle the nomenclature or nosology of OI. For example, if the gene-based classification were to be followed, then OI types I, II, III, and IV would be lumped into two groups—one due to mutations in COL1A1 and the other due to mutations in COL1A2. Almost all of the individuals with mutations in other genes were clinically classified into one of the first four groups. An alternative to this classification is to use one that builds on the clinical features and then subdivide the groups based on mode of inheritance and underlying gene. For example, OI type II, the perinatal lethal group, could have dominant forms the result from mutations in COL1A1 and COL1A2 and recessive forms that result from mutations in CRTAP, LEPRE1, and PPIB.

Type	Clinical characteristics	Inheritance ^a	Gene(s) involved	Type of mutation
I	Blue sclerae, normal or near-normal stature, no DI, hearing loss common	AD	<i>COL1A1</i>	Allele deletion Premature termination codons Assembly defects
II	Perinatal lethal	AD	<i>COL1A1</i> <i>COL1A2</i>	Substitutions for glycine in the triple-helical domain Exon skipping
III	Progressive deforming, short stature, DI	AD	<i>COL1A1</i> <i>COL1A2</i>	Substitutions for glycine in the triple-helical domain Exon skipping
IV	Normal sclerae, short stature, bone deformity	AD	<i>COL1A1</i> <i>COL1A2</i>	Substitutions for glycine in the triple-helical domain Exon skipping
V	Short stature, bone deformity, hyperplastic callus, mineralization of the radial-ulnar interosseous web	AD	<i>Unknown</i>	Unknown
VI	Normal facies and teeth, dramatic and progressive long-bone deformity and scoliosis	AR	<i>SERPINF1</i>	Missense mutations and nonsense mutations
VII	Broad range from perinatal lethal to a rhizomelic form with short stature and fractures	AR	<i>CRTAP</i>	Null mutations in the severe, the remainder have some protein made
VIII	Broad range from perinatal lethal to a clinical picture like OI type IV	AR	<i>LEPRE1</i>	Null mutations in the severe, the remainder have some protein made
IX	Range from perinatal lethal to an OI type IV picture	AR	<i>PPIB</i>	Null mutations in the severe, the remainder have some protein made
	Bruck syndrome—fracture and contractures	AR	<i>PLOD2</i> <i>FKBP10</i>	Most of the PLOD2 mutations are missense Most of the FKBP10 mutations are nulls

^aAD: autosomal dominant; and AR: autosomal recessive.

Bifid uvula: its clinical associations and underlying causes

Bifid uvula is an uncommon finding seen in approximately 1%–2% of apparently normal children (Shprintzen *et al.* 1985). It is, in some individuals, a sign of potentially life threatening aortic aneurysms. These individuals have mutations in one of the genes *TGFBR1* and *TGFBR2* that encode the TGF β receptor proteins on the

cell surface (Loeys *et al.* 2006). This condition, now known as Loeys–Dietz syndrome, was originally recognized by Mizuguchi and colleagues (2004), including Catherine Boileau, who had previously identified a large family with a Marfan-like phenotype in which genetic linkage studies excluded the involvement of the *FBN1* gene on chromosome 15 (Boileau *et al.* 1993; Collod *et al.* 1994; Mizuguchi *et al.* 2004). Mutations in both genes can lead to a spectrum of changes that include aortic dilatation, dissection,

and rupture at a young age, and hypertelorism and cleft palate (Loeys *et al.* 2006). At the other end of the spectrum, which can be seen in the same family, is the clinical picture of familial aortic aneurysm with dissections that may not manifest until the sixth and seventh decades (Pannu *et al.* 2005). Thus, in these families, recognition of the seemingly benign clinical sign of a bifid uvula could lead to a life-saving intervention.

In contrast to Loeys–Dietz syndrome, individuals with Marfan syndrome have a very narrow and often very high palate and significant crowding of both primary and secondary teeth because of the small size of both the mandible and maxilla. Aortic involvement is common in individuals with Marfan syndrome and is the most common reason for premature death. When recognized early, many of the aortic complications can be avoided by careful monitoring, the use of appropriate medications and timely surgical intervention, bringing life expectancy from the mid-30s to nearly normal. Sometimes it is the early recognition of palatal anomalies and dental crowding that bring these individuals into the broader medical world.

Back to clinical relevance

In our example here, George B. has already established good medical care and his general health is excellent. He had an echocardiogram done previously (because of the rare association of OI with aortic involvement); his aortic root diameter was normal, and there was no concern about an aortic aneurysm. Thus, his bifid uvula was likely to be a low-frequency normal variant. The major issue for him was preservation of his dental integrity. For the moment, use of resilient veneers could provide both a cosmetic and functional assist. There is a risk of tooth fracture, but this probably is not increased by the simple application of veneers. If there is persistent degradation, and for teeth already in poor shape, dental implants have been used. For George, the quality of bone, in general, in most regions of the mandible and maxilla is good enough to hold the implants, although it is best to have the procedures managed by an experienced practitioner, preferably one who has cared for individuals with OI and DI previously. Creating an environment in which George's dental care is handled as a matter of course is important and assures that he does not find himself marginalized.

Heritable connective tissue disorders in perspective: a wrapup

Heritable disorders of the genes that encode the proteins of the extracellular matrix are uncommon individually

but in aggregate could affect close to 1 in 500 individuals. The detailed study of the mechanisms of these disorders has lead to striking biological insights into the overall functions of these genes and their encoded products. While some clinicians will see patients with heritable connective tissue disorders only during training in tertiary care environments, others will encounter them in practice; the clinician's ability to recognize these patients could prove to be the link to therapies that will fundamentally alter their clinical course.

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SECTION 2

Teeth

14

Tooth development

Irma Thesleff and Emma Juuri

The process of tooth development is well understood in great detail. The main histological aspects were described more than a century ago. Studies by experimental embryologists from the 1960s to the 1980s revealed the key functions of tissue interactions and cell communication in the regulation of tooth morphogenesis and cell differentiation. Over the last two decades, the advances in gene technology methods, particularly with transgenic mice, allowed the analysis of the genetic and molecular mechanisms of tooth development. At present, dozens of genes and molecules are known that are necessary for the initiation and morphogenesis of teeth as well as for the formation of the dental hard tissues. The operations of genes in complex regulatory networks and their influence on cellular functions are increasingly well understood.

In order to appreciate the pathogenesis of dental aberrations and to design novel methods for their diagnosis, prevention, and treatment, it is important to understand the mechanisms of tooth development. It is already possible to induce the formation of new teeth in mice, and further advancements in research may lead to novel and improved techniques that will allow biological replacement of teeth in humans.

Developmental anatomy

Teeth develop as appendages from embryonic ectoderm. Their early morphogenesis shares similar anatomical features with other ectodermal appendages such as hairs and glands. In mammals, teeth form from the epithelium covering the first branchial arch and frontonasal prominence, and from the neural crest–derived mesenchyme underlying the epithelium. Before the initiation of the development of individual teeth, a thickened epithelial

stripe, the primary dental lamina, forms at the sites of the future dental arches of the maxilla and mandible. Subsequently, the dental placodes are formed within the dental lamina. Dental placodes are multilayered epithelial condensations resembling the placodes of other ectodermal organs both morphologically and functionally (Mikkola 2007). The placodal epithelium then forms a bud around which the neural crest–derived mesenchymal cells condense (Figure 14.1).

The transition from the bud to the cap stage begins when the epithelial bud invaginates at its tip. The enamel knot forms at this location as an aggregation of epithelial cells. The flanking epithelium grows down, forming the cervical loops. The mesenchymal cells that become surrounded by the epithelium form the dental papilla. These events determine the extent of the tooth crown. The epithelium differentiates into distinct cell layers that together form the enamel organ. These cells include the inner and outer enamel epithelia surrounding the stellate reticulum cells. The peripheral cells of the condensed dental mesenchyme give rise to the dental follicle that surrounds the enamel organ and dental papilla, and to the periodontal tissues.

During the following bell stage, the tooth germ grows rapidly and the shape of the tooth crown becomes evident. Secondary enamel knots form as epithelial cell aggregates, specify the points of epithelial folding, and determine the location of the cusps. The terminal differentiation of the tooth-specific secretory cells begins during this stage. The mesenchymal cells of the dental papilla, directly underlining the dental epithelium, differentiate into odontoblasts laying down the organic matrix of dentin. The juxtaposed epithelial cells differentiate into ameloblasts that deposit the enamel matrix. Cell differentiation and matrix deposition always start at

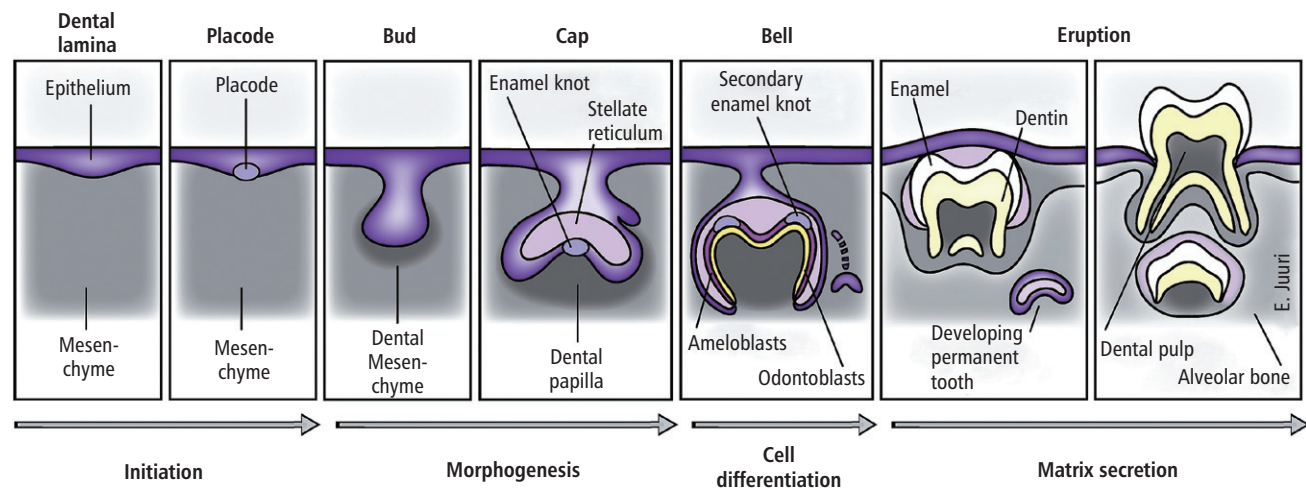


Figure 14.1 The main stages of tooth development. Interactions between the oral ectoderm and neural crest–derived mesenchyme direct morphogenesis and cell differentiation. The tooth shape results from growth and folding of the epithelial sheet. The epithelial signaling centers in the dental placode, and enamel knots are key regulators of morphogenesis.

the tips of future cusps marked by the sites of the secondary enamel knots. During the entire morphogenesis of the tooth crown, a gradient is seen where the stage of differentiation decreases from the cuspal tip toward the cervical area.

The root formation begins when the crown formation is complete. Root morphogenesis is guided by the growth of the cervical part of the dental epithelium. The stellate reticulum disappears from the cervical loop, and the inner and outer enamel epithelia come into contact, forming Hertwig's epithelial root sheath (HERS). Root growth is regulated by interactions between HERS and dental mesenchyme. HERS cells proliferate, but they do not differentiate into ameloblasts. Therefore, no enamel is formed in the root area. Instead, the HERS disintegrates and forms the epithelial cell rests of Malassez (ERM) that remain as a network on the root surface. The dental follicle cells coming into contact with root dentin differentiate into cementoblasts. The rest of the dental follicle cells in the root area form the periodontal ligament linking the tooth to alveolar bone.

The tooth erupts into the oral cavity soon after root development begins. At this time, the enamel layer of the tooth crown is still covered with the epithelial cells of the enamel organ. These are removed apoptotically as the tooth pushes its way into the oral cavity. At the time of eruption, the teeth are surrounded by bone. Therefore, the eruption depends on precisely regulated bone remodeling (i.e., bone resorption between crown and the oral cavity, and bone apposition at the base of the roots).

Replacement teeth form from the (secondary) dental lamina associated with the lingual aspect of the enamel organ of the deciduous tooth. The initiation of replace-

ment tooth development is evident as an offshoot from the enamel organ of the cap–bell stage deciduous tooth germ. This structure subsequently elongates in a cervical direction, and later buds to give rise to the replacement tooth (Figure 14.1; Luckett 1993; Järvinen *et al.* 2009). The extent of the dental lamina growth preceding the replacement tooth budding appears to vary between different teeth.

The period during which human teeth develop lasts an extremely long time. The first deciduous teeth are initiated during the fifth week of gestation, and their mineralization starts during the fourteenth week. At this time, the first permanent teeth have reached the bud stage and begin to mineralize prior to birth. The first deciduous teeth normally erupt in children at age six months, and the first permanent teeth at 5–6 years. The last teeth to be formed, the third molars, are initiated postnatally and their crown development is completed between 12 and 16 years. Hence, tooth germs representing many stages of development are present in the jaws of fetuses and children.

Currently, most of our knowledge of the developmental anatomy of teeth, particularly their developmental genetics, comes from studies using mice. As the central feature of the morphogenesis of individual teeth and their genetic regulation is remarkably similar across species, the information derived from mice is readily applicable to humans. The mouse dentition, however, differs from that of humans in some aspects. Mice completely lack cuspids and premolars, have only two incisors in the maxilla and mandible, and their incisors grow continuously. In addition, mice have only one dentition which restricts their use in studies of tooth replacement.

Cellular and molecular mechanisms of tooth development

Epithelial-mesenchymal interactions in tooth development

The odontogenic fate is programmed very early in the cells that will form the teeth. This was demonstrated in classical embryological studies decades ago. When the tissue in the molar area of a mouse embryonic jaw was dissected before the appearance of the first molar tooth buds and transplanted to the anterior chamber of the eye, all three molars developed (Lumsden 1988). Tooth buds were also shown to undergo morphogenesis when cultured as explants *in vitro*, indicating that morphogenesis does not depend significantly on vascularization, innervation, or interactions with bone and other surrounding tissues. Hence, teeth are determined at an early developmental stage, and, thereafter, the tissue committed to tooth development has inherent developmental potential.

Interactions between the epithelial and mesenchymal tissues play key roles in the regulation of tooth development. It has been shown in many experimental studies that when the dental epithelial and mesenchymal tissues were isolated and cultured in combination with various other tissues, the interactions are sequential and reciprocal:

there is a chain of interactive events between the two tissues driving advancing tooth morphogenesis (Kollar & Baird 1969; Mina & Kollar 1987; Ruch 1987; Lumsden 1988). At an early stage, the tooth-forming potential resides in the oral epithelium and shifts to the underlying mesenchyme around the time of placode formation. In addition to the interactions between the two tissue types, important signaling takes place between cells within each tissue. For example, the signaling molecule ectodysplasin (Eda) mediates communication exclusively between epithelial cells in developing teeth.

Cell and tissue interactions regulate a variety of cellular functions contributing to the formation of mesenchymal condensates and to the growth and folding of the epithelium. Such cellular functions include the division and migration of cells as well as cell adhesion and shape changes. The differentiation of odontoblasts and ameloblasts is regulated by epithelial-mesenchymal interactions as well, and the regulated cellular events include the exit from the cell cycle, polarization of the cells, and secretion of extracellular matrices of dentin and enamel.

Molecules participating in tooth development

Most data on the molecules regulating tooth development are derived from gene expression studies using *in situ* hybridization and immunohistology (Figure 14.2).

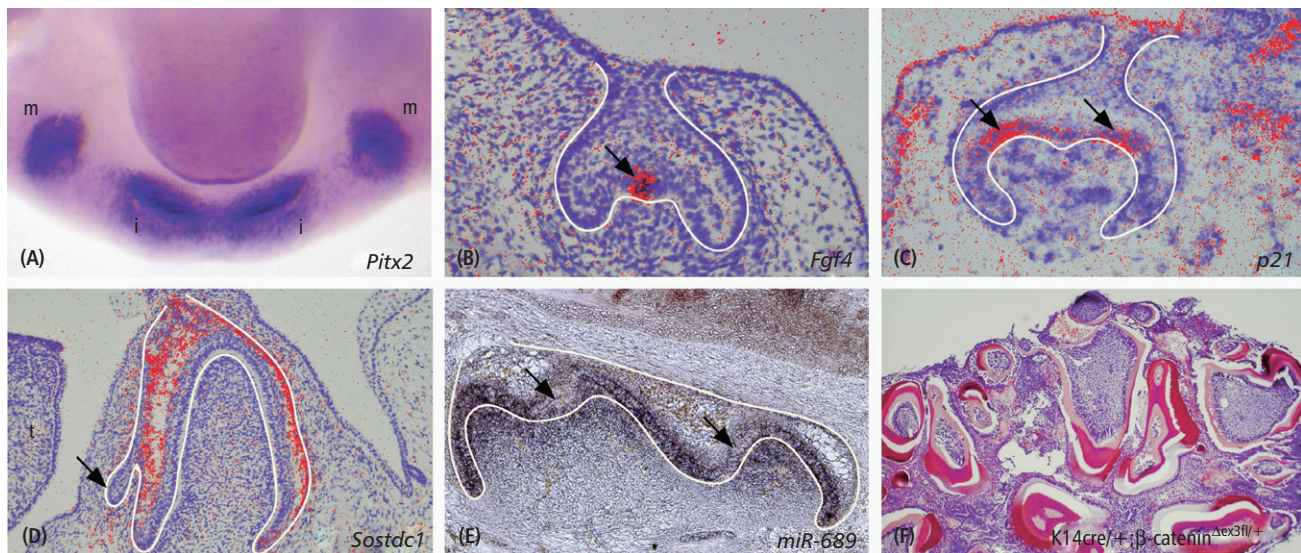


Figure 14.2 A. Incisor and molar placodes in E12 mouse mandible visualized by Pitx2 expression. m: molar; and i: incisor. B. Enamel knot in a cap stage molar of E14.5 mouse embryo visualized by Fgf4 expression. C. Secondary enamel knots in E16 bell stage mouse molar, visualized by p21 expression. D. Deciduous premolar of E34 ferret embryo. Sostdc1 (Ectodin) is expressed in the stellate reticulum compartment of the enamel epithelium. Formation of the permanent premolar is initiated from the outer enamel epithelium at the lingual side (arrow). t, tongue. E. miRNA-689 is intensely expressed in the inner enamel epithelium of the bell stage (E18) mouse molar, but it is excluded from the secondary enamel knots (arrows). F. Numerous teeth developed from one tooth germ of a transgenic mouse embryo in which Wnt signaling is activated in oral epithelium (K14cre/+; β -catenin $^{\Delta ex3fl/+}$). The tooth germ was grown for three weeks as a transplant in an adult mouse kidney. (Figure 14.2A and 14.2B are courtesy of Maria Jussila.)

Expression patterns of over 350 developmentally regulated genes can be viewed in the graphical database at <http://bite-it.helsinki.fi> (Tooth and Craniofacial Development Group 2007). The genes encode a variety of extracellular growth factors and matrix molecules, plasma membrane receptors, and cytoplasmic and nuclear proteins such as signal mediators and transcription factors, and micro-RNAs (miRNAs; Figure 14.2E). Expression data have revealed developmentally significant co-expression patterns of genes in various dental cell populations suggestive of gene regulatory networks. It is noteworthy that so far, no regulatory gene unique to teeth has been identified. In other words, all genes that are currently known to regulate tooth formation have functions in other developmental processes as well.

The majority of the genes in the database are associated with signaling networks mediating cell communication. Signaling molecules belonging to multiple families are expressed during tooth formation, and their receptors, mediators, and downstream targets have been localized to specific sites and cell types in developing teeth. Of particular importance for dental development are Tgf β , Activin, and bone morphogenetic proteins (Bmps) of the Tgf β superfamily; various molecules from the fibroblast growth factor and Wnt families; and sonic hedgehog (Shh) of the Hedgehog family (Thesleff 2003). As signaling molecules of these same conserved signal families have widespread functions in the regulation of embryonic morphogenesis and differentiation, it has

become obvious that the molecular basis of cell communication during tooth morphogenesis is based on the same toolkit of molecules as in all other embryonic organs.

Dental placodes and enamel knots as signaling centers

Placodes as well as primary and secondary enamel knots, the transient signaling centers appearing in the dental epithelium during early development, are characterized by the co-expression of several signaling molecules from all four conserved families. The primary enamel knots have been most extensively studied: at least 13 different signaling molecules including Shh and several Fgfs, Wnts, and Bmps have been localized in them (Figure 14.2; Thesleff 2003). Representatives of all four families also regulate the signaling functions of the placodes and secondary enamel knots. This is evidenced by the expression of many receptors and targets of the different pathways in the placodes and enamel knots (Figures 14.2 and 14.3). Thus, the different signal pathways converge in the signaling centers.

As described in this chapter, the reiterative appearance of epithelial signaling centers marks critical stages of tooth development. The dental placodes in the incisor and molar regions of embryonic day (E) 12 mouse embryos precede epithelial budding and initiate epithelial morphogenesis. The primary enamel knots appear during the transition from the bud to the cap stage and

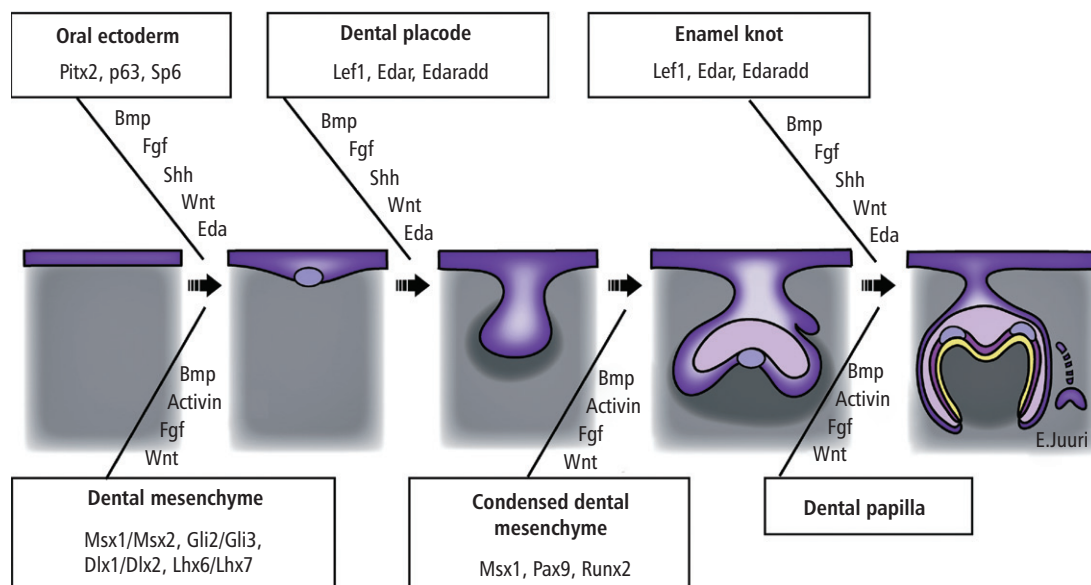


Figure 14.3 Schematic presentation of signaling networks mediating sequential and reciprocal interactions between the dental epithelium and mesenchyme. The same signaling molecules regulate development at all stages. The genes indicated in the boxes are necessary for normal tooth morphogenesis as shown by knockout mice studies. Mutations in several of these genes cause oligodontia in humans. Bmp: bone morphogenetic protein; Fgf: fibroblast growth factor; Shh: sonic hedgehog; and Eda: Ectodysplasin.

initiate the morphogenesis of the tooth crowns. They regulate proliferation in the underlying mesenchyme forming the dental papilla, and in the flanking epithelium forming the cervical loops. The secondary enamel knots precede the folding of the inner enamel epithelium and initiate cusp formation (Figures 14.1 and 14.3).

Careful three-dimensional comparisons of gene expression patterns and tooth morphologies have revealed close associations between the primary and secondary enamel knots and tooth shapes, and have indicated central roles for the enamel knots in patterning the tooth cusps. Interestingly, a wide variety of tooth shapes in different mammalian species and cusp patterns can be reproduced by mathematical modeling of enamel knot signaling (Jernvall & Thesleff *in press*).

The ectodysplasin (Eda) signal pathway has a specific role in the regulation of dental signaling centers (Mikkola 2009). The Eda receptor Edar is specifically expressed in tooth placodes and enamel knots. It is also expressed in the placodes of other ectodermal organs, and mutations in the Eda pathway cause the human hypohidrotic ectodermal dysplasia syndrome (HED) manifesting deficient formation of all ectodermal organs. The mouse mutants lacking the expression of Eda, Edar, or Edaradd (a cytoplasmic mediator of Eda signal) are models for human HED. They often lack the third molars or incisors and have small molars with cusp-patterning defects characterized by fused and lacking cusps. The phenotype is due to small placodes and primary enamel knots as well as fused secondary enamel knots (Pispa *et al.* 1999). In line with this, mice overexpressing Eda in the ectoderm have large molar placodes and enamel knots resulting in the development of extra premolar-like teeth in front of the first molars and abnormal molar cusp patterns (Mustonen *et al.* 2004). These features, together with the demonstration that Eda recombinant protein stimulates placode growth, indicate that Eda signaling regulates the activity of the signaling centers (Mikkola 2009). Interestingly, downstream targets of the Eda pathway include signals and inhibitors of all four conserved signal pathways (Fliniaux *et al.* 2008; Mikkola 2009; Zhang *et al.* 2009). Thus, Eda is an important modulator of the major signaling pathways in the placodes and enamel knots.

Gene regulatory networks

A general picture of the gene networks regulating tooth morphogenesis has emerged based on studies from many laboratories carried out during the 1990s and 2000s (Figure 14.3; Thesleff 2003). A variety of tooth defects reported in transgenic and mutant mice include missing and supernumerary teeth, defects in dental hard tissues, and abnormalities in tooth size and shape. Detailed analyses of tooth phenotypes have provided

data on the gene regulatory networks operating during tooth development. In most cases, the modified genes have been associated with signal pathways. Thus, dental defects may result from the impaired function of signaling molecules, receptors, modulators, mediators, or targets in signaling pathways.

The complete block of any of the four conserved signaling pathways leads to an early arrest of tooth formation. For a review, see Tummers and Thesleff (2009) and Bei (2009b). Examples of this are the deletion of either Fgf8 or Bmp receptor 1a in oral epithelium, overexpression of the Wnt inhibitor Dkk1 in the epithelium, and deletion of Shh mediators Gli2 and Gli3 (Figure 14.3). The loss of function of a variety of transcription factors that are targets of the signaling pathways results in arrested tooth formation as well. The factors include Pax9, Pitx2, Runx2 (targets of Fgf pathway), Lef1 and β -catenin (targets of Wnt pathway), and Msx1 and Dlx2 (targets of Bmp pathway). In some cases, a double knockout of transcription factors in the same family is required indicating redundant functions. Examples are Msx1 and Msx2, Dlx1 and Dlx2, and Lhx6 and Lhx7. It is also noteworthy that in most knockouts, tooth development is arrested either prior to dental placode development and budding (E11) or prior to primary enamel knot formation and transition to the cap stage (E13).

The downstream targets of signaling molecules have been examined in *in vitro* studies by introducing recombinant signaling molecules to dissected dental tissues and detecting gene expression by *in situ* hybridization and RT-PCR (Närhi *et al.* 2010). The use of tissues from mouse mutants in these assays has allowed further dissection of the gene regulatory networks. For example, such experiments have revealed the role of epithelial and mesenchymal Bmp4 at the early stage of tooth development and positioned mesenchymal Msx1 both upstream and downstream of Bmp4 (Vainio *et al.* 1993; Bei & Maas 1998). In addition, Msx1 is regulated by epithelial Fgfs, but this pathway is distinct from the Bmp4 pathway. Here Msx1 as well as Runx2, the gene mutated in cleidocranial dysplasia syndrome, are required for the induction of Fgf3 by epithelial Fgfs (Bei & Maas 1998; Åberg *et al.* 2004). Furthermore, Wnt signaling is integrated in this network as indicated by the requirement of Lef1 for the expression of Fgf4 in the enamel knot and β -catenin for the expression of Fgf3 in the mesenchyme (Kratochwil *et al.* 2002; Chen *et al.* 2009).

The fine-tuning of signaling pathways during morphogenesis is important, and the localized activity of specific inhibitors of signaling families is essential for normal development (Tummers & Thesleff 2009). For instance, follistatin, which antagonizes activin and Bmp signaling, is required for normal tooth crown

development. The patterning of secondary enamel knots in follistatin knockout mice is impaired, resulting in irregular folding of the dental epithelium and abnormal cusp pattern (Wang *et al.* 2004). Molar cusp patterning is also affected in mice deficient for the Wnt and Bmp inhibitor *Sosdc1* (also known as *ectodin* and *Wise*). These mice have extra teeth in front of the first molars (Kassai *et al.* 2005; Ahn *et al.* 2010). Similar supernumerary premolar-like teeth are caused by the modulation of other signal pathways, including *Eda* (Mustonen *et al.* 2003), *Fgf* (Klein *et al.* 2006), and *Shh* (Ohazama *et al.* 2009). The most recent signaling pathway modulators associated with tooth development are miRNAs that are posttranscriptional regulators of gene expression. The expression of many miRNAs targeting signaling pathway components is developmentally regulated in teeth. Furthermore, the impairment of miRNA processing in the dental epithelium, by deleting the function of *Dicer*, results in dental defects including increased tooth number, crown shape abnormalities, and defects in ameloblast differentiation and enamel structure (Cao *et al.* 2010; Michon *et al.* 2010).

The differentiation of ameloblasts and odontoblasts at the interface between the epithelium and mesenchyme is regulated by epithelial-mesenchymal interactions. The extracellular matrix, particularly the basement membrane, is important for the polarization and differentiation of the hard tissue-producing cells. In addition, several signaling pathways have been implicated in these processes (reviewed by Ruch *et al.* 1995; Bei 2009a): Bmps induce ameloblast and odontoblast differentiation, *Shh* is required for amelogenesis, and active Wnt signaling is present in the differentiating ameloblasts and odontoblasts, suggesting functions in their regulation (Suomalainen & Thesleff 2010).

Tooth replacement and regeneration

Tooth replacement and regeneration are associated with signal pathway activities, particularly those of the Wnt pathway. Although the mouse teeth are normally not replaced, the stimulation of Wnt signaling in oral epithelium induces the formation of numerous extra teeth (Figure 14.2F; Järvinen *et al.* 2006; Liu *et al.* 2008; Nakamura *et al.* 2008; Wang *et al.* 2009). When the mutant tooth buds were cultured in vitro, new teeth formed continuously. It was concluded that the activation of Wnt signaling may have unlocked the potential for tooth renewal that mice have lost through evolution (Järvinen *et al.* 2006). Evidence for the inductive role of the Wnt pathway in de novo tooth formation also comes from a hereditary disorder in humans called *familial adenomatous polyposis* (FAP) which is associated with a predisposition to colorectal cancer. This syndrome is caused by

APC gene mutations resulting in activation of the Wnt pathway and, in some cases, supernumerary teeth and odontomas containing multiple small teeth. Interestingly, a network of Wnt, Hedgehog, and Bmp signaling regulates tooth replacement in snakes (Handrigan & Richman 2010).

It is obvious that the replacement and regeneration of teeth require the existence of stem cells in the dental tissues. Intriguingly, putative stem cells were localized during tooth replacement in the gecko on the lingual aspect of the dental lamina (Handrigan *et al.* 2010), which thus represents the same region in which mammalian replacement teeth are initiated (Luckett 1993; Järvinen *et al.* 2009). It is unfortunate that mice, which are the best models in the study on tooth development, do not replace their teeth. However, dental stem cells have been located and studied in mice in the continuously growing incisors. They appear to reside in a niche that bears anatomical similarities to the well-studied stem cell niches of hair and intestine (Harada *et al.* 1999; Seidel *et al.* 2010). The maintenance and differentiation of the epithelial stem and progenitor cells of the incisor are regulated by epithelial-mesenchymal interactions mediated by conserved signaling molecules. Fgfs act in a reciprocal regulatory loop together with mesenchymal *ActivinA* and *Bmp4*. *Shh* and *Notch* signaling also participate in the maintenance and early differentiation of the epithelial stem and progenitor cells (Harada *et al.* 1999, 2002; Wang *et al.* 2007; Klein *et al.* 2008; Parsa *et al.* 2010; Seidel *et al.* 2010). A more thorough understanding of the molecular signatures of stem cells as well as of epithelial and mesenchymal cell lineages and their interactions will be crucial for successful bioengineering of teeth in the future (Thesleff & Tummers 2009).

Conclusions, medical and dental implications, and future perspectives

Greater knowledge of the following eight considerations of the mechanisms of tooth development is instrumental in understanding the pathogenesis of human dental defects and for designing methods for treatment and bioengineering of teeth.

1. Congenital dental aberrations are often manifestations of many malformation syndromes. This is understandable because the genetic and cellular mechanisms of development are very similar in all organs. The fact that the most common such syndromes are ectodermal dysplasias affecting two or more ectodermal organs is conceivable since teeth develop as ectodermal appendages, all of which are initiated from placodes and share molecular and cellular mechanisms.

2. Clinical studies have shown that the defects in tooth number, size, and shape are often linked in human conditions and that smaller teeth, peg-shaped incisors, and molars with fewer cusps are associated with hypodontia. This is conceivable since the same genes are used reiteratively during morphogenesis, and their mutations may affect several stages of tooth development. In particular, deficient signaling from dental placodes initiating tooth development or from enamel knots regulating the crown size and cusp patterning can explain why tooth agenesis and morphological defects are linked.
3. Since the placodes and enamel knots express many molecules in different signaling pathways, it is conceivable that mutations in many different genes may affect both the number and shape of teeth.
4. It is well established that human hypodontia affects certain teeth more often than others. This may be explained by the development of teeth in each family from a common placode and by the fact that the most frequently missing teeth develop as the last teeth of a family (incisors and molars). The size of the dental placode may determine a graded threshold for the number of teeth developing from that placode (Nieminen 2009). Therefore, the last teeth that develop from the placode (such as the third molars and lateral incisors) would be the most vulnerable to developmental disturbances and would be more severely affected than the teeth developing earlier.
5. Defects in dental hard tissues are sometimes associated with aberrations in the number and/or shape of teeth. This is conceivable because the same genes, particularly genes associated with signal pathways, are used for the regulation of morphogenesis as well as the differentiation of odontoblasts and ameloblasts.
6. Because genes function in networks and several genes regulate the same cellular functions, mutations in many different genes may cause a similar tooth phenotype. This implies that it is difficult or even impossible to predict which gene is mutated in a specific tooth defect.
7. The ultimate goal of the research on the mechanisms and genetic basis of tooth development and regeneration and the pathogenesis of dental defects is to generate knowledge that could be applied in clinical practice to diagnosis, prevention, and treatment of the defects. Although the prevention of dental defects will probably not be possible in the majority of cases, prevention may be feasible in certain specific cases. Intriguingly, injections of *Eda* protein can correct the tooth phenotype of *Eda* mutant mice and dogs, representing animal models for X-linked HED (Casal *et al.* 2007). These experiments are wonderful exam-

ples of research that begins with the identification of a human disease gene which then can lead to potential clinical applications.

8. After optimal sources of stem cells for tooth bioengineering have been discovered, knowledge of the genetic, molecular, and cellular mechanisms of normal tooth development will be crucial for growing teeth. The regeneration of teeth—or any other organ—from a single population of stem cells is not likely to be feasible because the interactions of two tissues of different origins, the epithelium and mesenchyme, are crucial for all aspects of tooth formation.

Summary

Teeth develop from oral epithelium and underlying neural crest–derived mesenchyme. Morphogenesis and cell differentiation are regulated by interactions between epithelial and mesenchymal tissues mediated by conserved signaling molecules that regulate the expression of numerous genes. Signaling centers in the dental epithelium play key roles: the placodes initiate tooth formation, and the enamel knots regulate crown development. Studies using genetically modified mice indicate that gene defects in all major signaling pathways can cause tooth agenesis, morphological aberrations, and defects in hard-tissue formation. Because the same genes that regulate tooth morphogenesis are needed for the development of other organs and tissues, dental anomalies are often found in syndromes, mostly associated with defects in hairs, glands, and other ectodermal organs. Tooth replacement and regeneration have recently been linked with signaling pathway activities. Understanding of the genetic, molecular, and cellular mechanisms of tooth development will form the basis for tooth bioengineering.

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Clinical correlate: tooth agenesis

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Tooth development is genetically controlled and disturbances in this process can lead to tooth agenesis affecting mostly the permanent dentition. There are several environmental factors such as viral infections, toxins, and radio- or chemotherapy that may disrupt normal tooth formation. However, most disruptions are believed to be caused by genetic factors. Since many of the genes that are involved in tooth development also direct morphogenesis of other organs, tooth agenesis can be syndromic, meaning that it may be associated with additional, syndrome-specific malformations or dysfunctions involving other organs.

The heritability of congenitally missing teeth has been documented in many studies. The inheritance pattern may be dominant, recessive, or X-linked, and it appears that in some cases several genetic factors may act together, with or without contributions from environmental factors. The importance of genetic factors is shown not only by familial clustering but also by higher concordance in identical than in non-identical twins. Sometimes the tooth agenesis condition is overlooked in family members because it manifests only as smaller than normal and/or peg-shaped teeth. Some hypodontia patients have recessive tooth agenesis, which manifests only in people who inherit one copy of the tooth agenesis gene variant from each of their unaffected parents. In X-linked tooth agenesis, only some of the male family members show the condition. It is also possible that a tooth agenesis patient has no family history, because a new mutation was acquired through the parents' germ cells or during early embryogenesis.

To date, only four nonsyndromic tooth agenesis genes (PAX9, MSX1, AXIN2, and EDA) have been identified and analyzed (Mues *et al.* 2009; Nieminen 2009). The pattern of missing teeth provides an important clue.

PAX9 mutations lead mostly to molar agenesis, while MSX1 mutations affect more premolar teeth; selective tooth agenesis caused by changes in the EDA gene present with incisor agenesis, and AXIN2 mutations are responsible for severe mixed agenesis. However, there must be many more causative genes because most families with congenitally missing teeth do not have a mutation in any of these four genes. Thus, if genetic tests for mutations in the four identified genes are negative, it does not mean that the patient's condition is caused by environmental factors but that the patient has a mutation in one of the as yet unidentified genes.

There are several reasons for trying to identify tooth agenesis genes. One is to gain better insights into the processes that promote tooth development—knowledge that will advance our proficiency in bioengineering of dental tissue. Another reason is the development of causative tooth agenesis treatments for patients. This is not a far-fetched idea: It has been shown that EDA protein injections at an early age can prevent tooth agenesis and other ectodermal dysplasia symptoms in EDA-deficient dogs (Casal *et al.* 2007). Recombinant EDA protein for treatment of humans with ectodermal dysplasia and EDA-caused hypodontia is now under development. In addition to substitution of the mutated protein, there are other means of treatment such as pharmacological modulation of pathway activity in which a mutated protein operates or a nonsense mutation suppresses.

Case presentation 1

A family with autosomal dominant tooth agenesis affecting mostly molars

A 13-year-old boy was seen by an orthodontist for evaluation of agenesis of several permanent posterior teeth.

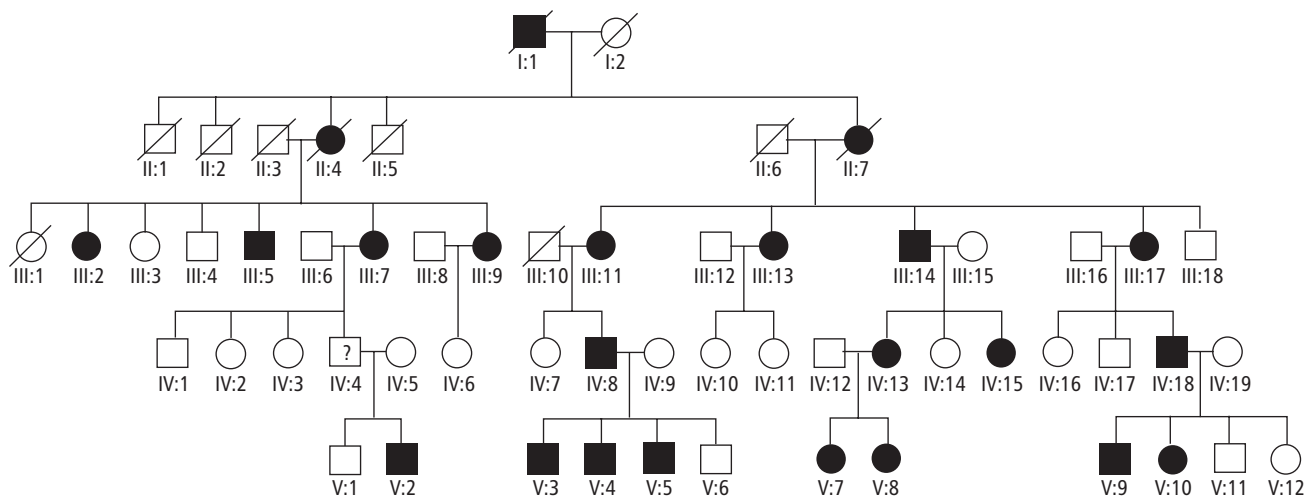


Figure 15.1 Pedigree of a family (Case 1) with agenesis of predominantly posterior permanent teeth. Affected males and females are represented as filled squares and circles, respectively. All affected family members carried a 219insG frameshift mutation in the *Pax9* gene.

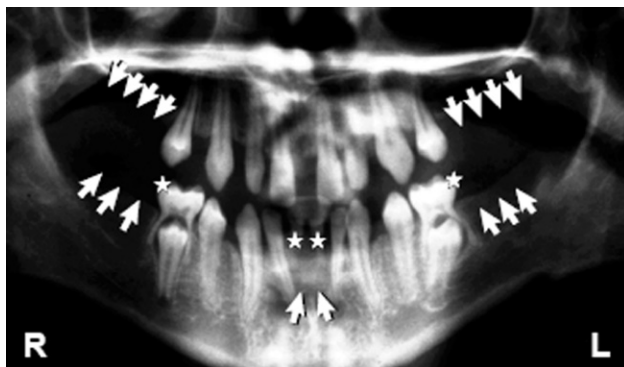


Figure 15.2 A panoramic radiograph of the dentition of a 13-year-old individual V:5; arrowheads indicate missing teeth, and stars indicate persistent primary teeth.

His father, his paternal grandmother, two of his brothers, and several second-degree aunts, uncles and cousins were similarly affected; another brother had a normal dentition. History, X-rays, and an oral exam of father and son revealed that both were congenitally missing all of their permanent molars, their maxillary second premolars, and one or two mandibular central incisors. The father's mandibular second and maxillary first premolars were also absent. There were no other medical or morphological problems.

The family agreed to participate in a genetic study for the detection of the causative gene. After obtaining informed consent, a pedigree was established, and the phenotype of all affected family members was documented (Figures 15.1, 15.2, and 15.3).

DNA was isolated from blood and cheek swab samples. Since the family was quite large and all of the members

Right quadrants				Left quadrants			
Molars	Premolars	Can	Incisors	Incisors	Can	Premolars	Molars
Upper 3 2 1	2 1	1	2 1	1 2	1	1 2	1 2 3
Lower 3 2 1	2 1	1	2 1	1 2	1	1 2	1 2 3
IV:8	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★
IV:13	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★
IV:18	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★
V:2	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★
V:3	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★
V:4	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★
V:5	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★
V:7	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★
V:8	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★
V:9	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★
V:10	★ ★ ★	★ ★	★ ★	★ ★	★	★ ★ ★	★ ★ ★

Figure 15.3 Synopsis of the permanent dentition in Case 1's affected family members. Filled stars represent absent teeth.

were willing to participate, linkage analysis was performed pointing to chromosome 14q12-q13 as the location of the faulty gene. Of all of the genes in this region of chromosome 14, the *Pax9* gene was the most likely candidate responsible for the missing tooth problem since it has been implicated in early tooth development. All four exons of the *Pax9* gene were PCR amplified. Sequencing of the PCR products revealed heterozygosity for an insertion of a G nucleotide at position 219 of the *Pax9* coding region in each of the affected family members but not in the unaffected members (Stockton

et al. 2000). The 219 G insertion leads to a frameshift early on in Pax9 mRNA translation resulting in a non-functional protein. Since a mutation in only one of the two Pax9 alleles appears to be sufficient for producing the hypodontia phenotype, the tooth agenesis phenotype is most likely caused by haploinsufficiency reflecting the sensitivity of the developmental processes to fluctuations in Pax9 gene expression. A 50% reduction in gene dosage is usually well tolerated.

Treatment included genetic counseling to explain that, on average, half of the children of each affected person would be affected and there may be some variability in the number and location of affected teeth reflecting the different genetic background of each individual.

Case presentation 2

A family with X-linked tooth agenesis affecting mostly incisors

A Caucasian male with missing maxillary lateral and all mandibular incisors was encouraged by his wife to participate in a genetic tooth agenesis study. The couple had one unaffected daughter, but the participant had two

affected brothers, an uncle, a cousin, and four nephews presenting with missing incisors excepting maxillary central incisors; some missing first premolars were also noted. All affected individuals reported that their primary dentitions had a similar pattern of missing incisors. The family had no signs of any systemic disorders.

The pedigree pattern of this family (Figures 15.4 and 15.5) strongly suggested an X-linked inheritance in that only males were affected and all of the affected males had phenotypically normal parents and offspring. The most likely candidate gene in this family was the EDA gene, which is located on the X-chromosome and causes the syndrome known as *hypohidrotic ectodermal dysplasia* as well as nonsyndromic, selective tooth agenesis.

DNA from cheek swabs and blood samples was PCR amplified with primers to each of the eight EDA coding exons and then sequenced. All affected males had a T-to-C mutation in exon 8, changing amino acid 365 from valine to alanine. The mothers and daughters of the affected males were carrier females, meaning that they were heterozygous for the mutation but phenotypically unaffected because their second X chromosome compensates for the mutated allele. In vitro functional

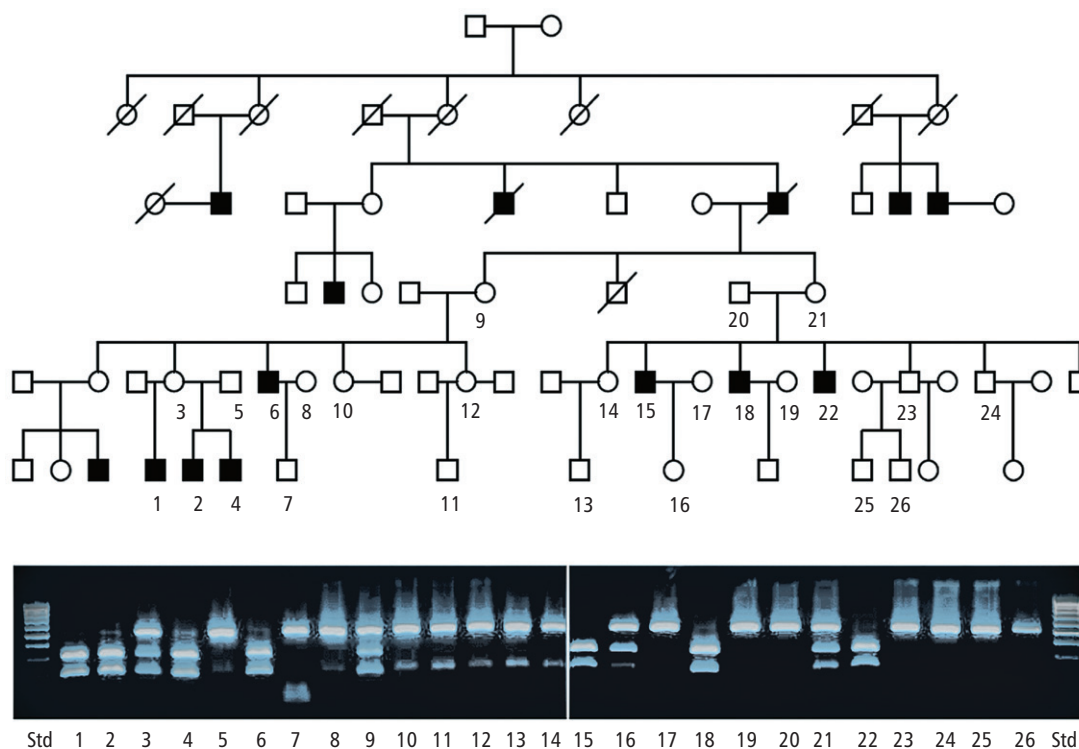


Figure 15.4 Pedigree and phenotype of a family (Case 2) with selective tooth agenesis of mostly anterior teeth caused by an EDA gene mutation. The pedigree showed an X-linked inheritance pattern (upper panel) and segregation of the mutation with affected and carrier status (lower panel; one band: unaffected family members; two bands: affected males; and three bands: carrier females). Numbers 1–26 denote the family members for whom DNA samples were available. Open circle: healthy female; open square: healthy male; filled square: affected male; and “/”: deceased.

	Primary teeth	Permanent teeth
1	5 4 3 1 1 3 4 5 5 4 3 2 1 1 2 3 4 5	8 7 6 5 3 1 1 3 5 6 7 8 8 7 6 5 4 3 2 1 1 2 3 4 5 6 7 8
2	5 4 3 1 1 3 4 5 5 4 3 2 1 1 2 3 4 5	Too young
4	5 4 3 1 1 3 4 5 5 4 3 2 1 1 2 3 4 5	Too young
6	5 4 3 1 1 3 4 5 5 4 3 2 1 1 2 3 4 5	8 7 6 5 3 1 1 3 5 6 7 8 8 7 6 5 4 3 2 1 1 2 3 4 5 6 7 8
15	5 4 3 1 1 3 4 5 5 4 3 3 4 5	7 6 5 4 3 1 1 3 4 5 6 7 7 6 5 4 3 3 4 5 6 7
18	5 4 3 1 1 3 4 5 5 4 3 2 3 4 5	8 7 6 5 4 3 1 1 3 4 5 6 7 8 8 7 6 5 4 3 2 3 5 6 7 8
22	5 4 3 2 1 1 3 4 5 5 4 3 2 3 4 5	8 7 6 5 4 3 2 1 1 3 5 6 7 8 6 5 4 3 2 3 4 5 6

Figure 15.5 Tooth agenesis pattern in Case 2's affected males. Incisors are most frequently absent except for maxillary central incisors. For primary teeth, 1: central incisors; 2: lateral incisors; 3: canines; and 4–5: molars. For permanent teeth, 1: central incisors; 2: lateral incisors; 3: canines; 4–5: premolars; and 6–8: molars. Missing numbers indicate missing teeth.

studies showed that protein function of the V365A EDA protein was reduced but not completely abolished similar to the ectodermal dysplasia-causing mutations in the same gene (Mues *et al.* 2010).

Genetic counseling for X-linked disorders is often difficult because of the complicated inheritance pattern: (1) X-linked disorders affect mainly males because they have only one X chromosome; (2) all daughters of affected males are carrier females because they inherit their father's affected X chromosome; (3) since sons inherit only the Y chromosome from their father, they will never be affected; (4) affected males are born to unaffected parents, but the mothers are carriers and may have affected male relatives; (5) on average, 50% of the sons of carrier females are affected and 50% of the daughters are carriers; and (6) females can be affected if the father is affected and the mother is a carrier or because of nonrandom X inactivation in a carrier female. The family with the V365A mutation was also alerted to follow the development of recombinant EDA for human treatment.

Case presentation 3

A family with multiple polymorphisms in the *Axin2* gene

Tooth agenesis caused by *Axin2* gene mutations (Figures 15.6, 15.7, and 15.8) appear to be quite rare. However, the detection of *Axin2*-caused tooth agenesis is very

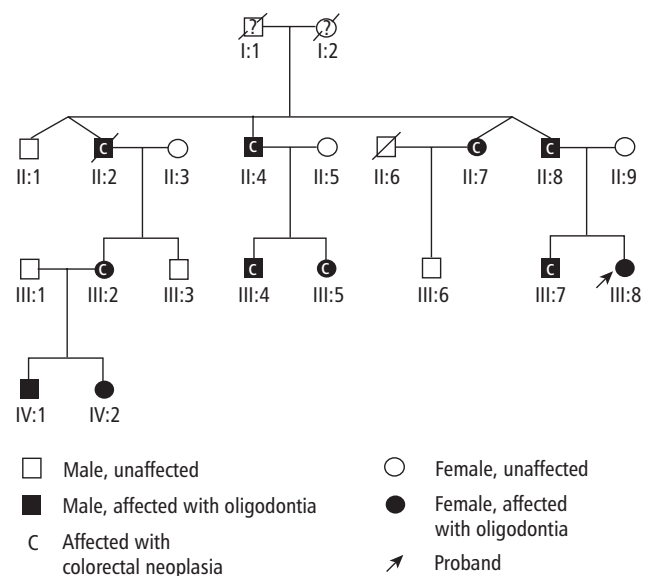


Figure 15.6 Pedigree of a four-generation family (Case 3) showing autosomal dominant inheritance of oligodontia phenotypes and colorectal neoplasia and agenesis of permanent teeth caused by *AXIN2* mutations.

important because these patients are high-risk candidates for developing colon cancer (Lammi *et al.* 2004). Thus far, two mutations have been shown to cause both tooth agenesis and colon cancer. Several other *Axin2* mutations have been classified as polymorphisms because they are found in more than 1% of the popula-

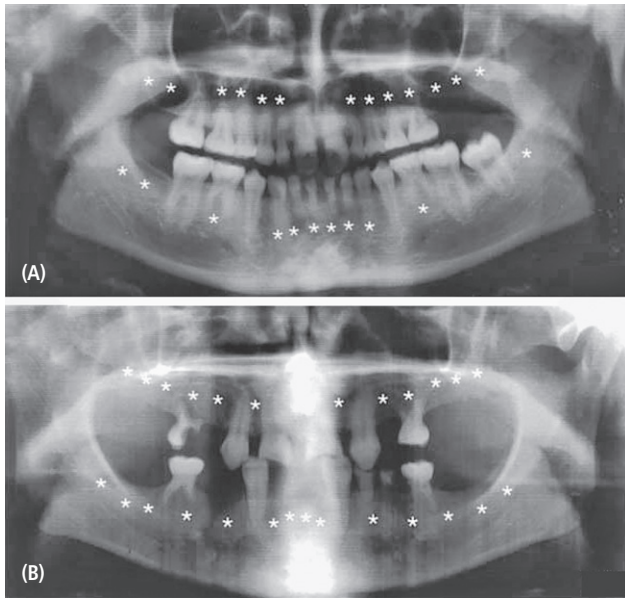


Figure 15.7 Panoramic radiographs of the proband (Case 3) at age 15 years (A) and her uncle at age 34 years (B). Stars indicate congenitally missing permanent teeth. Note that both family members have many persisting deciduous teeth.

tion and are not associated with any consistently recognizable phenotypes. There are, however, reports that some *Axin2* polymorphisms are found more frequently in people with missing teeth than in people without tooth abnormalities (Mostowska *et al.* 2006).

A family consisting of an unaffected father, a mother with missing lateral incisors, and three teenage children with more severe tooth agenesis entered the study for genetic evaluation with special attention to *Axin2* gene mutations. The 13-year-old girl had only missing lateral incisors, missing third molars, and a rotated canine. The two boys, ages 15 and 16 years, were missing nearly all of their teeth except for the maxillary central incisors and first molars.

According to the available information, there were two distant relatives with lateral incisor agenesis and two relatives with a cleft lip and palate on the mother's side of the family. The mode of inheritance appeared to be autosomal dominant with incomplete penetrance and/or variable expression. The *Pax9*, *Msx1*, *EDA*, and *Axin2* genes were sequenced, but no mutations were detected except for polymorphisms in the *Axin2* gene. The father and all three children were heterozygous for the two *Axin2* polymorphisms that are more common in tooth agenesis patients. It is conceivable that these *Axin2* polymorphisms contribute to the phenotype of the children by aggravating the effects of an as yet unknown causative gene from the mother's side of the family. It is unlikely, however, that these *Axin2* polymorphisms are a major

causative factor since the father and many other people with these polymorphisms do not suffer from tooth agenesis. *Axin2* polymorphisms have not been associated with susceptibility to colon cancer, providing some reassurance to the parents.

The family was not suitable for linkage analysis, which is the procedure that would allow delineation of the chromosomal location of the causative gene. Instead, the identification of the causative gene is being pursued by a candidate gene approach that consists of direct sequencing of all genes that are known to be involved in tooth development and that cause missing teeth in genetically modified mice.

Conclusion

Understanding genetic factors associated with oral craniofacial abnormalities is important to the field of dentistry. A solid knowledge of the genetic basis of pathological dental conditions is needed both for patient counseling and for providing state-of-the-art patient treatment. Similar to the development of a whole organism, the morphogenesis of teeth is strictly controlled by a genetic program; the size, number, shape, patterning, eruption, and material composition of teeth are largely determined by genes. Small variations in the sequence or expression of these genes can have a great impact on the formation of the dentition, its durability, and its function. Exact knowledge of the developmental process and its constituent genes will make it possible to find causative treatments for developmental dental malformations without having to resort to gene therapy. In addition, since many of these genes are also involved in the development of other organ systems, specific dental abnormalities can signal susceptibility to disease or malformation in other organ systems connecting dentistry to general medicine.

Summary

Congenital tooth agenesis is very common and ranges from frequently missing third molars to the very rare absence of all teeth (anodontia). Both extremes and every phenotype in between are governed by individual variations in gene structure and expression patterns during odontogenesis. Currently we are only able to detect fairly crude malfunctions like gene deletions and mutations of single genes. But patients are already benefitting from our knowledge of the underlying cause of their conditions through genetic counseling and early detection of possible associated cancer susceptibilities. Furthermore, there are realistic prospects for the development of cures for these conditions by non-invasive and non-genome-altering means.

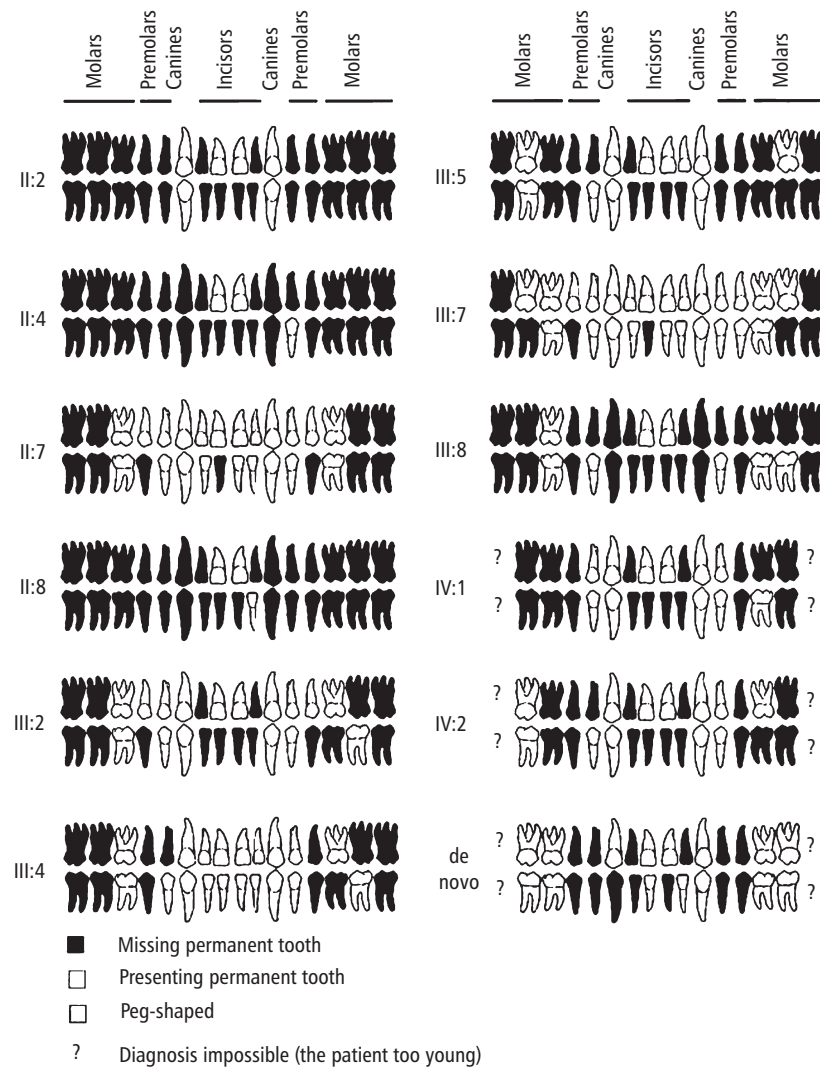


Figure 15.8 The number of missing permanent teeth (Case 3) ranged from 8 to 29. Agenesis of deciduous teeth was found in only one individual (IV1; not shown).

Acknowledgments

We would like to thank all of the participating families and Dr. Jeff Karp for referring family number 3.

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16

Dentin

Chunlin Qin and Jian Q. Feng

The vertebrate tooth is made of four types of tissues: enamel, dentin, cementum, and pulp. The former three are mineralized tissues, while pulp is a nonmineralized tissue. Enamel is derived from the ectoderm, while the remaining three types of dental tissues develop from the ectomesenchyme originating from the neural crest cells. In the crown, dentin and enamel are bound together through the dentinoenamel junction, while in the root, dentin and cementum are connected at the cementodentinal junction. Unlike enamel, dentin is a vital and sensory tissue which responds to stimuli and is continuously formed after tooth formation is complete.

Although dentin is a hard tissue and pulp is a soft tissue, they are related embryologically, histologically, and functionally. Therefore, dentin and pulp are often described together as the *dentin–pulp complex*. The pulp is the nonmineralized, loose connective tissue that occupies the pulp cavity in the central portion of the tooth. The pulp cavity, in the crown portion called the *pulp chamber*, conforms to the shape of the crown. The pulp cavity in the root is referred to as the *root canal* that terminates at the apical foramen, through which the pulp and periodontal ligament communicate. The nerve fibers, blood vessels, and lymphatic vessels enter and leave the pulp via the apical foramen. In addition to longitudinal root canals that travel from the bottom of the pulp chamber to the apical foramen, a tooth may contain lateral canals that provide connections between the pulp and the periodontal ligament along the lateral surface of the root. The communications between the pulp and periodontal ligament provide nutrition for the pulp and serve as routes for the spreading of infections from one to another. The odontoblast bodies in the pulp

form a layer that lines a zone of nonmineralized type I collagen matrix known as *predentin*. Predentin is converted to dentin when hydroxyapatite crystals are deposited on predentin. Dentin and predentin matrices contain multiple closely packed dentin tubules that transverse the entire thickness of dentin and predentin, and contain odontoblast processes. The major components in the matrices of dentin and predentin include hydroxylapatite, type I collagen, noncollagenous proteins (NCPs), proteoglycans (PGs), and water.

Dentin formation

The formation of dentin follows the same principles that guide the formation of other hard connective tissues in the body, namely, cementum and bone. In the formation of dentin, odontoblasts synthesize and secrete type I collagen-rich unmineralized extracellular matrix (ECM) (i.e., predentin), which is subsequently mineralized. As the odontoblasts build this type I collagen-rich fibrillar matrix, they recede in a pulpal direction and leave behind odontoblastic processes, through which these cells remain connected to the mineralized matrix. Formation of predentin and its transformation to dentin as mineralization takes place comprise a highly controlled, orderly process. For example, under normal conditions of growth, the predentin width is rather uniform, not random, indicating that the rates of synthesis of the unmineralized matrix and its conversion to dentin at the predentin–dentin border must be equal. On the other hand, in pathological conditions like dentinogenesis imperfecta (DGI), the widening and disorganization of the predentin layer indicate an incapacitation of this process.

Differentiation of odontoblasts

In dentinogenesis, the first requirement is the presence of highly specialized cells termed *odontoblasts*. Odontoblasts are differentiated from neural crest cell–derived ectomesenchymal cells present in the dental papilla during tooth development. During the formation of a tooth, cells (ameloblasts) in the inner dental epithelium deliver signals to the undifferentiated ectomesenchymal cells that are adjacent to the inner dental epithelium. The undifferentiated ectomesenchymal cells receiving the signals from the ameloblasts will undergo a number of steps, enlarging and elongating to become preodontoblasts first and then odontoblasts that contain large amounts of protein-synthesizing organelles. The odontoblasts are highly polarized, with the nuclei positioned away from the inner dental epithelium.

The differentiation of ectomesenchymal cells into odontoblasts needs growth factors and transcription factors (Thesleff 2003). In the initial stage of dentinogenesis, the inner dental epithelium delivers bone morphogenetic proteins (BMPs belonging to the TGF β superfamily) and fibroblast growth factors (FGFs) to induce the ectomesenchyme into odontogenic mesenchyme (Thesleff & Mikkola 2002). BMPs and FGFs induce the expression of a number of mesenchymal transcription factors, many of which are needed not only for dentinogenesis but also for the continuation of tooth development as a whole. The necessity of transcription factors for tooth development is well illustrated by the observation that tooth agenesis may result from the double mutants of *Msx1* and *Msx2*, and *Dlx1* and *Dlx2*, and in the *Pax9* null mice (Thesleff 2003). In addition to the TGF β and FGF families, several members in the conserved Hedgehog and Wnt families, acting as paracrine signal molecules, also play crucial roles in the differentiation of ectomesenchymal cells into odontoblasts. These factors regulate interactions between the ectoderm and mesenchyme; they also mediate communication within the mesenchyme (Thesleff 2003). Working collectively, these factors drive ectomesenchymal cells into differentiated odontoblasts responsible for making dentin.

Matrix proteins and the mineralization of dentin

Although extensive studies on dentinogenesis have not elicited a thorough understanding of the exact roles that each of the dentin ECM molecules plays, some valuable clues have been obtained and speculations about their functions have been formulated to address the questions regarding the formation of dentin. For example, ultrastructural studies on the collagen fibrils formed by rat

incisor odontoblasts demonstrated that collagen fibrils progressively thicken from the time they are secreted until they are mineralized at the predentin–dentin border (Beniash *et al.* 2000); these observations along with many others (Yamauchi *et al.* 1992; Septier *et al.* 1998) indicate that the transition from the zone of predentin immediately outside the bodies of the odontoblasts to the area adjacent to dentin represents a gradient of events, a maturation process that is dynamic. Another example is the important discovery that mutations in the dentin sialophosphoprotein (DSPP) gene are responsible for the underlying dentinal defects seen in dentinogenesis imperfect (DGI) type II and type III (Xiao *et al.* 2001; Zhang *et al.* 2001), which are characterized by severe defects in dentin mineralization that affect a large number of individuals worldwide. In DGI type II and type III, both the deciduous and permanent teeth are clinically affected, appearing blue-gray or amber brown and opalescent.

More than 90% of the organic components in dentin matrix are type I collagen. The importance of the correct collagen structure in dentin formation is clearly seen in patients with DGI type I caused by mutations in the type I collagen gene (Kim & Simmer 2007), which clinically resemble DGI type II and III caused by DSPP mutations. In addition to type I collagen, the ECM of dentin contains a number of NCPs and proteoglycans (Table 16.1). One category of the NCPs that are principally found in dentin and bone and are secreted into the ECM during the formation and mineralization of these tissues is termed the SIBLING (small integrin-binding ligand, n-linked glycoprotein) family that includes DSPP, dentin matrix protein 1 (DMP1), bone sialoprotein (BSP), and osteopontin (OPN; Fisher *et al.* 2001). Data obtained in the last several decades have demonstrated that these polyanionic SIBLING proteins are actively involved in the mineralization of collagen fibers and crystal growth within predentin, when this tissue is converted to dentin. The remainder of this section will provide an updated summary of the four SIBLING family members and their postulated roles in the formation and mineralization of dentin.

DSPP

Gene mutation studies in humans (Xiao *et al.* 2001; Zhang *et al.* 2001) and investigations involving *Dspp* gene knockout mice (Sreenath *et al.* 2003) have demonstrated that DSPP is crucial for dentinogenesis. Dentin in *Dspp*-null mice is hypomineralized and the predentin is widened (Figure 16.1), giving rise to a phenotype similar to the manifestations of DGI types II and III in humans.

Table 16.1 Noncollagenous proteins and proteoglycans present in the matrix of dentin. *Category:* Noncollagenous components are classified into the SIBLING family and the proteoglycan category. Those that cannot be classified into either of the two groups (such as osteocalcin and osteonectin) are simply listed as noncollagenous proteins (NCPs). *Quantity:* The quantity described here reflects the amount of an individual component, relative to other members in the same category. For example, DSP-PG is the most abundant one in the proteoglycan category, so it is listed as a major component although it is relatively much less than DPP. *Function:* If the functions of a component are not well defined, the function column is marked “unclear.” DSP and DPP are the cleaved products of DSPP. DMP1-37 kDa and DMP1-57 kDa fragments are the cleaved products of DMP1. DSP-PG is the proteoglycan form of DSP. DMP1-PG is the proteoglycan form of DMP1-37 kDa fragment.

	Category	Quantity	Potential functions in dentin
DSP	SIBLING	Major	Unclear
DPP	SIBLING	Major	Initiate and regulate mineralization
DMP1-37 kDa	SIBLING	Moderate	Unclear
DMP1 57 kDa	SIBLING	Moderate	Nucleate hydroxyapatite
BSP	SIBLING	Minor	Nucleate hydroxyapatite
OPN	SIBLING	Minor	Inhibit and regulate mineralization
MEPE	SIBLING	Minor	Inhibit and regulate mineralization
DSP-PG	Proteoglycan	Major	Unclear
DMP 1-PG	Proteoglycan	Major	Unclear
Biglycan	Proteoglycan	Minor	Unclear
Decorin	Proteoglycan	Minor	Get involved in collagen fibrinogenesis
Osteocalcin	NCPs	Major	Unclear
Osteonectin	NCPs	Moderate	Unclear

The large precursor protein, DSPP, predicted from cDNA (MacDougall *et al.* 1997; Prasad *et al.* 2010) gives rise to two proteins: dentin sialoprotein (DSP) with coding sequences in the 5′ end and dentin phosphoprotein (DPP) representing the 3′ end of the DSPP mRNA. The occurrence of one gene transcribing a single mRNA encoding DSP and DPP indicates that the translated product, DSPP, must be cleaved by a proteinase giving rise to the individual proteins, DSP and DPP. Recent studies indicate that DSPP is cleaved into DSP and DPP by bone morphogenetic protein 1 (von Marschall & Fisher 2010). In fact, only minor amounts of full-length DSPP are present in the ECM dentin; an overwhelming majority of full-length DSPP is cleaved into NH₂-terminal and COOH-terminal fragments (Sun *et al.* 2010). DPP, discovered about four decades ago, is the most abundant NCP in dentin ECM. The unusual feature of DPP is the occurrence of large amounts of acidic residues such as aspartic acid and phosphoserine. Data obtained through in vitro mineralization studies indicate that DPP is an important initiator and modulator for the formation and growth of hydroxyapatite (HA) crystals (Boskey *et al.* 1990). Following its synthesis and secretion by odontoblasts, DPP is transported to the

mineralization front, where it binds to collagen fibrils and assumes a conformation that promotes the formation of initial HA. As the mineralization process proceeds and predentin is converted to dentin, these mineral crystals grow in an oriented fashion under the modulation of DPP and other NCPs that bind to the growing HA faces (Butler & Ritchie 1995). DSP, discovered about three decades ago, is another abundant NCP in dentin ECM. The functions of DSP are presently undefined; it has little or no effect on in vitro mineralization. Recently, a proteoglycan form of DSP (referred to as DSP-PG) has been isolated and characterized (Yamakoshi *et al.* 2005; Zhu *et al.* 2010).

The ECM of dentin, therefore, contains four molecular variants derived from DSPP amino acid sequence: DSP, DPP, DSP-PG, and a minor amount of full-length DSPP. It is likely that these variants that vary greatly in biochemical structure play different roles during dentinogenesis. As stated above, DPP is known to be an initiator and modulator for the formation and growth of hydroxyapatite, whereas information regarding the biological roles of DSP and DSP-PG is lacking. Based on (1) the presence of DSP and DPP in dentin ECM, (2) the absence of significant amounts of DSPP, and (3) the

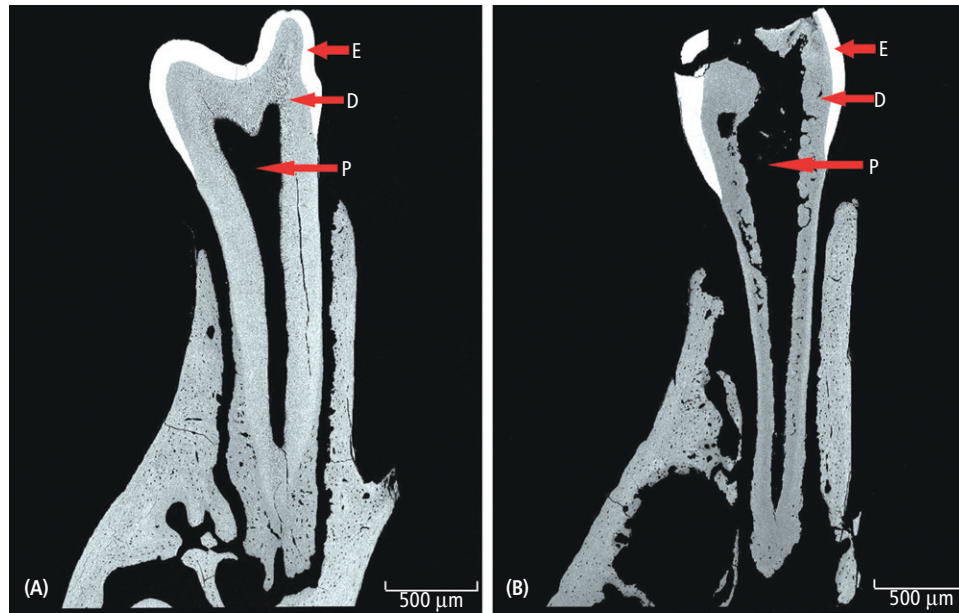


Figure 16.1 Loss of DSPP leads to defects in dentin formation. Back-scattered scanning electron microscope (SEM) images of the first mandibular molar of a three-month-old wild-type mouse (A) and the first mandibular molar of a three-month-old *Dspp*-null mouse (B). Note the thinner dentin and enlarged pulp chamber in the molar of the *Dspp*-null mouse. The defects in dentin formation are due to a failure in the conversion from predentin to dentin. (*Dspp*-null mice were obtained from the Mouse Mutant Regional Resource Center, MMRR#:000047-UNC; the back-scattered SEM images were generated by Monica Prasad, Texas A&M Health Science Center, Baylor College of Dentistry Department of Biomedical Sciences. Used with permission.)

observed roles of DPP in the nucleation and modulation of apatite crystal formation, it is likely that the conversion of DSPP to DSP and DPP is an activation event, converting an inactive precursor to active fragments, as in the case of zymogen activation. This activation step would represent one of the controlling mechanisms in dentin formation (Prasad *et al.* 2010). The correct structure for DSP (within DSPP) may be required for proper folding or transport of DSPP. The folding and/or structure of this large precursor DSPP may prevent formation of the three-dimensional structure that would allow DPP to bind calcium or to collagen, in a manner prescribed for it to initiate and control mineralization. Although it is reasonable and based on all the evidence at hand, this visualization of the role and activation of DSPP is lacking in direct, supportive evidence.

DMP1

DMP1 is an acidic phosphoprotein primarily expressed in dentin and bone (George *et al.* 1993; Qin *et al.* 2007). The distinctive feature of DMP1 is the presence of a large number of acidic domains, a property that implicates it as a possible participant in regulating matrix mineralization. The importance of DMP1 for dentin mineralization has been demonstrated by knockout experiments in mice and human genetic studies: *Dmp1*-null mice show

profound dental defects including widening of predentin and hypomineralization of dentin (Figure 16.2), while mutations in the human *DMP1* gene are associated with osteomalacia and rickets, which affect the formation and mineralization of bone and dentin (Jiang *et al.* 2010). Like DSPP, DMP1 is present in the ECM of bone and dentin as (1) a NH₂-terminal (37 kDa) fragment, (2) a COOH-terminal (57 kDa) fragment, (3) a proteoglycan form (DMP1-PG) of the NH₂-terminal fragment, and (4) a minor amount of the full-length form. These four forms, differing dramatically in biochemical structure, may have different functions in dentinogenesis and osteogenesis. Based on (1) DMP1 existing as processed fragments in the ECM of dentin and bone, (2) the absence of significant quantity of full-length DMP1, and (3) the observed roles of 57 kDa fragment in mineralization (Qin *et al.* 2007), it is reasonable to believe that the proteolytic conversion of DMP1 to its corresponding fragments may be an activation step, releasing active forms at the correct time and site to control the mineralization process of dentin and bone.

BSP

The primary sequence of BSP was first deduced from a rat cDNA sequence (Oldberg *et al.* 1986, 1988). BSP consists of much fewer amino acid residues than DSPP or DMP1. The biological functions of BSP in mineral-

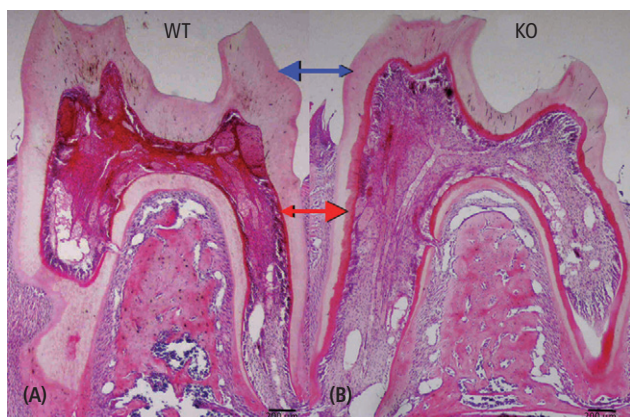


Figure 16.2 Loss of DMP1 leads to defects in dentin formation. A. H&E staining of the first mandibular molar of a one-month-old wild-type mouse. B. The first mandibular molar of a one-month-old *Dmp1*-null mouse. The layer of mineralized dentin (indicated by blue arrows) in the molar from the *Dmp1*-null mouse (KO) is thinner than the wild type (WT), suggesting a slower rate of dentin formation in the mutant. In contrast, the predentin layer (between black arrows) is wider in the molar from the *Dmp1*-null mouse than the wild type, indicating a slower rate in the conversion of predentin to dentin in the mutant. These findings indicate that when DMP1 is inactivated, the conversion of predentin to dentin is incapacitated. (This research was originally published in *J Biol Chem*. Ye, L., MacDougall, M., Zhang, S., Xie, Y., Zhang, J., Li, Z., Lu, Y., Mishina, Y. and Feng JQ. Deletion of Dentin Matrix Protein-1 Leads to a Partial Failure of Maturation of Predentin into Dentin, Hypomineralization and Expanded Cavities of Pulp and Root Canal During Postnatal Tooth Development. *J Biol Chem* 2004 30;279(18):19141–8 © the American Society for Biochemistry and Molecular Biology.)

ized tissues are largely unknown, although some data suggest that BSP acts as a nucleator for the formation of initial apatite crystals; then, as this mineral grows on the collagen matrix, it acts as an inhibitor in directing the growth of the crystals (Hunter & Goldberg 1993). The tissue distribution of BSP is relatively restricted to the mineralized tissues, primarily in bone. In the tooth, BSP can be observed in cementum and predentin, and under physiological conditions (primary and secondary dentinogenesis), mineralized dentin contains little or no BSP (Moses *et al.* 2006). In tertiary dentin, the level of BSP is remarkably elevated.

OPN

The name *osteopontin* was introduced to reflect the potential of this protein in bone to serve as a bridge between cells and hydroxyapatite through Arg–Gly–Asp tripeptide and polyaspartic acid motifs in its primary amino acid sequence discovered by cDNA cloning (Olderberg *et al.* 1986). OPN contains a similar number of

amino acids as BSP. Although OPN is present in bone in relatively large quantities, it is also expressed in a variety of other tissues and cells (Sodek *et al.* 2000); the broad expression of OPN indicates a multiplicity of functions in diverse biological events. Under physiological conditions, only minor amounts of OPN exist in the ECM of dentin (Qin *et al.* 2001). In tertiary dentin formation, the level of OPN is remarkably elevated. In mineralized tissues, OPN is believed to be an effective inhibitor of apatite formation and growth (Boskey *et al.* 2002).

Basic structure of dentin

Predentin

Predentin is the organic fibrillar matrix of the circum-pulpal dentinal matrix before its calcification into dentin. Predentin consists principally of collagen and noncollagenous components. Similar to osteoid in bone, predentin stains less intensively than mineralized dentin in hematoxylin–eosin-stained sections. Predentin gradually mineralizes into mineralized dentin as various NCPs are incorporated into the mineralization front. The thickness of predentin in a tooth remains constant because the amount that calcifies is balanced by the addition of new unmineralized matrix.

Mineralized dentin

Mature mineralized dentin is made up of approximately 70% hydroxyl apatite, 20% organic, and 10% water by weight (Nanci 2003). Dentin is harder than cementum and bone, but softer than enamel. The mineralized dentin has an elastic quality that is important for the proper function of the tooth because the elasticity provides flexibility and prevents fracture of the overlying brittle enamel. Root dentin is recognized as being structurally and compositionally different from crown dentin: the orientations of collagen fibers are different, the rate of deposition of root dentin is slower, and the DPP content is less than that of the crown dentin.

Dentinal tubules and odontoblast processes

The odontoblast processes, similar to osteocyte processes, run in canalicules that transverse predentin and dentin layer. These canalicules are called *dentinal tubules*, which extend through the entire thickness of the dentin and predentin from the dentinoenamel junction to the pulp and form a network for the diffusion of nutrients. In the process of caries, the dentinal tubules provide routes for rapid spreading of the pathogens.

Types of dentin

The dentin formed before the completion of the tooth formation is called *primary dentin*, while that formed

after the completion of tooth formation is designated *secondary dentin*. *Tertiary dentin* is the dentin formed by odontoblasts in response to external stimuli such as abrasion, dental caries, or the preparation of restorative cavities. Primary dentin and secondary dentin are physiological; these two types of dentin are nearly identical in both morphological appearance and chemical composition. Tertiary dentin, which is considered pathological dentin, differs greatly from primary dentin in both morphology and chemical composition (D'Souza *et al.* 1995; Moses *et al.* 2006); it is further classified into reactionary dentin and reparative dentin. If the odontoblasts receiving these harmful external stimuli, which are relatively mild and/or moderate, can survive, then the tertiary dentin formed by these existing odontoblasts is called *reactionary dentin*. If the noxious stimuli are strong enough to result in the death of odontoblasts, the tertiary dentin made by new odontoblasts differentiated from subodontoblasts is designated *reparative dentin*.

Conclusions and future directions

In the last several decades, investigators in the field of dentin research have attempted to answer the following questions: what is the exact composition of dentin matrix? What defects are present and which genes are responsible for the inherited dentin disorders? What is the nature of the ECM molecules that modulate the initiation, rate, and extent of dentin deposition? How do the physical features and conformational structures of ECM molecules facilitate the formation and mineralization of dentin? Do these macromolecules interact with each other during the mineralization process? Do the ECM molecules send feedback signals to the odontoblasts and influence the behaviors of these cells? What genes regulate the expression of key dentin ECM molecules? The efforts of many researchers have led to a better understating for some of the above questions. For example, we know now that the mutations in the DSPP gene cause dentinogenesis imperfecta type II and type III, while mutations in the type I collagen gene are responsible for dentinogenesis imperfecta type I. However, dentin formation is a dynamic and complicated process, involving interplays among a number of molecules including type I collagen, NCPs, and prtoteoglycans, which work collectively to precisely control the site and rate of apatite formation. Despite the efforts and progresses made in the past several decades, the process of dentin mineralization is still not well understood. The available data indicate that dentin matrix proteins play important roles in the mineralization of this tissue, although the exact mechanisms by which each individual molecule participates in biomineralization are unclear.

Clearly, there is a need to further explore the interactions among the molecules within the complex milieu of dentin ECM. Information from such studies will not only help to formulate the fundamental mechanisms about how dentin forms but also enhance understanding concerning the pathogeneses of dentin defects occurring in systemic diseases such as DGI and dentin dysplasia. A better elucidation of the pathogeneses underlying these dental defects is essential for establishing scientifically based treatment modalities for such diseases.

Summary

Dentin, the bulky tissue of the tooth body, is formed by the ectomesenchyme-derived odontoblasts. It is the mineralized tissue that provides protection to the underlying, nonmineralized pulp and lends support to the overlying, mineralized enamel. In the formation of dentin, type I collagen secreted by odontoblasts forms the scaffold upon which hydroxylapatite crystals are deposited. In addition to type I collagen, the extracellular matrix contains a number of noncollagenous proteins, which play critical roles in the initiation and regulation of hydroxyapatite crystals, the inorganic component of vertebrate mineralized tissues. Dentin differs from enamel in that (1) dentin is a vital tissue, which responds to external stimulus; (2) dentin formation is continuous throughout life; and (3) dentin is a tubular tissue. Dentin is similar to bone and cementum; these three types of mineralized connective tissues are vital and sensory tissues whose major organic components are type I collagen and noncollagenous proteins.

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Clinical correlate: dentinogenesis imperfecta, restorative procedures, and caries

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Dentin contains 70% minerals, 20% proteins, and 10% water by weight. Compared to dental enamel, dentin is softer and has lower mineral and higher organic contents. Odontoblasts responsible for dentin matrix secretion and mineralization are highly differentiated and specialized cells that are vulnerable when subjected to local or systemic disturbances. Unlike dental enamel, dentin is produced throughout the life span of the tooth, and upon stimulation dental pulp cells may differentiate into odontoblast-like cells and resume the function of dentin production and maintenance (Nanci 2008). Dentin defects, often manifested with discoloration and/or structural malformation, can be the result of genetic or environmental insults. Careful review of a patient's medical and dental history and assessment of clinical and radiographic evidence are key approaches to discerning the underlying etiology of dentin defects.

Dentin and dentin defects

Environmentally induced dentin defects often are the result of physical, chemical, or nutritional exposure that negatively impact the developing tooth germs and/or odontoblast functions. Depending upon the stage of odontogenesis, intensity, and duration, the disturbances of dentin formation may be evident in selected teeth of the primary and/or the permanent dentition. In contrast, heritable dentin defects impact all teeth in both the primary and the permanent dentitions, although the severity often varies between the primary and the permanent dentitions (Figure 17.1). This group of conditions has long been observed in human populations (Gray 1970); *hereditary opalescent dentin* was the term

used to describe inherited dentin defects that occur in the absence of systemic manifestations (Hodge 1936).

Classification of inherited dentin defects

Heritable dentin defects can be viewed as a continuum with a common denominator along which both the primary and the permanent dentitions are affected in an autosomal dominant trait. Shields and colleagues (1973) classified such defects into two categories: dentinogenesis imperfecta and dentin dysplasia. Clinically there are three types of dentinogenesis imperfecta—DGI-I (MIM 166200), DGI-II (MIM #125490), and DGI-III (MIM #125500)—and two types of dentin dysplasia, DD-I (MIM #125400) and DD-II (MIM #125420).

DGI-I

Patients with this disorder have osteogenesis imperfecta (OI). Systemically they experience bone fragility that is often associated with short stature, tooth abnormalities (dentinogenesis imperfecta), and blue sclera. The most common mutations associated with OI result from the substitution of glycine by another amino acid in the triple-helical domain of either the alpha-1 or the alpha-2 chain of collagen type I (Rauch *et al.* 2010). The primary and permanent teeth have marked discoloration and attrition. Pulpal obliteration may occur prior to or soon after tooth eruption. The dental phenotypic expression among patients with OI varies significantly.

DGI-II

This disorder has many clinical and radiographic similarities to DGI-I, but penetrance is often complete and

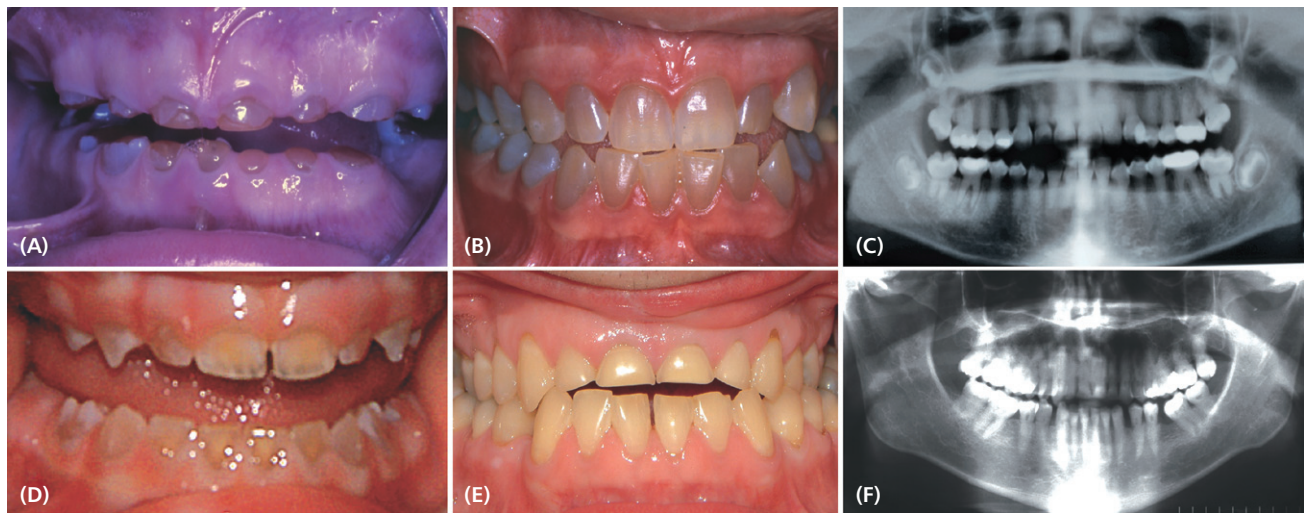


Figure 17.1 Phenotypic variations of patients with inherited dentin defects. Primary dentition (A), permanent dentition (B), and panoramic radiograph (C) of patient with DGI-II; primary dentition (D), permanent dentition (E), and panoramic radiograph (F) of patient with DD-II. Significant difference in the degree of discoloration between the primary and permanent dentition is evident in both conditions. Although a thistle-shaped pulp chamber with pulp stones is a hallmark of DD-II, complete pulp obliteration may take place over time after the eruption of permanent teeth in patients with DD-II, such as seen with the incisors (F).

expressivity is more consistent within a family when compared with DGI-I (Bixler *et al.* 1969). The classic radiographic features of DGI-II include bulbous crowns with cervical constriction, thin and narrow roots, and pulpal obliteration. DGI-II is one of most commonly encountered dominantly inherited disorders affecting approximately one patient in every 8000 (Witkop 1988).

DGI-III

This condition was first documented in the “Brandywine isolate” from southern Maryland and has an incidence rate of 1:100,000. The clinical hallmark is multiple pulp exposures observed in the primary dentition of affected individuals. Radiographically, the primary teeth show considerable variation in appearance, ranging from pulpal obliteration to shell teeth (Shields *et al.* 1973).

DD-I

In DD-I, the clinical crowns of both permanent and primary teeth can appear to be normal in shape, form, and color, but radiographically the teeth have short roots with a crescent-shaped pulpal remnant parallel to the cemento-enamel junction in the permanent dentition and total pulpal obliteration in the primary dentition. Based on the characteristics of the pulpal remnants, a subclassification of DD-I has been proposed. Increased mobility and multiple periapical radiolucencies without frank decay are frequently observed (Brenneise *et al.* 1989; Seow 1994).

DD-II

This condition has an incidence rate similar to that of DGI-II. The primary teeth share the same features as in DGI-II. The permanent teeth may have normal shape, form, and root length, although some or all of them may show variable grayish to brownish discoloration. The pulp cavities of developing or newly erupted permanent teeth show a thistle-tube deformity and frequently contain pulp stones (Shields *et al.* 1973; Brenneise & Conway 1999).

The overlapping clinical presentation of these conditions has made correct diagnosis challenging. More importantly, the distinguishing dental phenotypes of more than one type are commonly observed in different affected individuals from the same kindred (Heimler *et al.* 1985; Witkop 1988).

Genetics of dentin defects

Genetic analyses linked DGI-II (Ball *et al.* 1982; Aplin *et al.* 1995), DGI-III (Boughman *et al.* 1986), and DD-II (Dean *et al.* 1997) to the 4q21 region, making the SIBLING family the prime candidate genes for these disorders. Defects in the DSPP gene have been shown to cause DGI-II (Xiao *et al.* 2001; Zhang *et al.* 2001; Kim *et al.* 2004; Malmgren *et al.* 2004; McKnight *et al.* 2008), DGI-III (Kim *et al.* 2005; Song *et al.* 2006; Lee *et al.* 2009), and DD-II (Rajpar *et al.* 2002; McKnight *et al.* 2008). Although genes encoding the minor noncollagenous proteins in dentin, such as dentin matrix protein 1

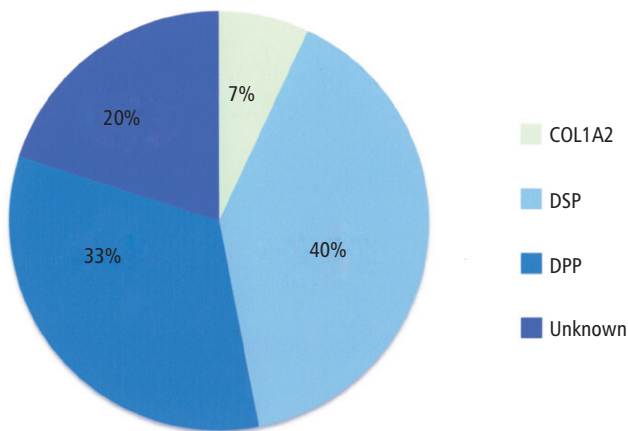


Figure 17.2 Percent chance of determining causative gene mutations based on a pilot investigation of 15 kindreds with inherited dentin defects. Mutational analyses of families with clinical diagnoses of DD-I, DGI-II, or DGI-III having no apparent skeletal abnormalities and using a target gene approach resulted in the identification of disease-causing mutations in COL1A2 (1 kindred), DSP (6 kindreds), and DPP (5 kindreds; unpublished data).

(DMP1), integrin binding sialoprotein (IBSP), matrix extracellular phosphoglycoprotein (MEPE), and secreted phosphoprotein-1 or osteopontin (SPP1, OPN) and members of the SIBLING family also are logical candidates, disease-causing mutations in those genes have not been identified among individuals with inherited dentin defects (Kim & Simmer 2007). Mutational analyses of candidate genes in 15 DD-II and DGI kindreds with no apparent skeletal abnormalities identified disease-causing mutations in COL1A2, DSP, and DPP (Figure 17.2), while analyses of the DD-I families have not yielded a positive outcome. Compared to amelogenesis imperfecta, the chance of identifying a causative gene mutation among families with dentin defects is higher (80% vs. 50%, unpublished data). Based on current evidence and understanding of the genetic etiology of dentin defects, it is important to consider that DD-II, DGI-II, and DGI-III represent a single disorder with increasing levels of severity (Beattie *et al.* 2006; Kim & Simmer 2007; McKnight *et al.* 2008).

Diagnosis, progression, and prognosis of inherited dentin defects

Diagnosis of this group of disorders is typically reached based on family history and on clinical and radiographic assessment. Many systemic conditions may present dental features resembling inherited dentin defects. A correct diagnosis not only is critical for treatment plan-

ning, but also allows for a timely referral should medical management become necessary. Differential diagnosis can rule out specific conditions including amelogenesis imperfecta with severe discoloration, congenital porphyria, hypophosphatemic rickets, intrinsic staining of the dentition due to medications, and conditions leading to premature tooth loss such as Chediak–Higashi syndrome, histiocytosis X, hypophosphatasia, and Papillon–Lefevre syndrome. In order to avoid the negative social and functional consequences of the disorders, early diagnosis and intervention are critical for preserving structure and optimizing prognosis (Coffield *et al.* 2005).

Abnormalities of the affected dentition that may require intervention include discoloration, attrition, pulpal obliterations, and tooth-shape variations. Delay or accelerated dental age has been reported in patients with dentin disorders (Larsson & Nordblom 1981). In the DGI-I group, the underlying OI subtype has the greatest impact on overall growth and development (O’Connell & Marini 1999; Muhney & Campbell 2007). Although caries associated with abraded first permanent molars, caries along the margin of restorations and root caries, have been reported in DGI/DD cases, there are no definitive data suggesting that there is a higher risk of caries development among affected individuals. Microhardness tests show that average readings for dentinogenesis imperfecta are approximately half those of normal dentin (Helmers & Finn 1966). Therefore, crown or crown–root fractures subsequent to trauma (Miers & Herbert 1990) or endodontic treatment are frequently encountered complications. Loss of vertical dimension in the load-bearing areas can lead to accelerated attrition in the incisal area, which affects the growth and development of the craniofacial complex including the temporomandibular joints. Investigations of malocclusion among subjects with osteogenesis imperfecta have shown that Class III dental malocclusions occurred in 70%–80% of this population, with a high incidence of anterior and posterior crossbite and open bite (O’Connell & Marini 1999).

A variety of treatment recommendations have been reported including placing crowns on essential or core teeth; providing veneers, composite restorations, or overdentures; placing dental implants; and providing preventive orthodontic treatment. Because of rapid attrition, treatment may be initiated at the primary and mixed dentition stage to maintain vertical dimension and arch length, to protect the permanent dentition, and to correct anterior teeth with conservative preparations for improving esthetics and function (Helmers & Finn 1966). Conventional treatment plans emphasize placing full coverage crowns on the first molars, followed shortly by restoration of the incisors followed by restoration of the canines,

premolars, and second molars as needed. Because of the treatment complexity, time commitment, guarded prognosis, and financial burden, it is not uncommon for patients to elect to have extractions and receive full dentures in early adulthood (Barron *et al.* 2008).

Based on a 2009 report of five cases with amelogenesis imperfecta or dentinogenesis imperfecta in Switzerland, a median of 24 teeth per patient was reconstructed with a total treatment cost of about \$20,000 per patient. Compared to patients with cleft lip and palate and patients with hypodontia or oligodontia, patients with amelogenesis or dentinogenesis imperfecta acquired the highest treatment costs and required the most maintenance and retreatment. A significant amount of initial treatment expense was due to periodontal interventions. Although the initial treatment costs per tooth were higher for implant cases, the eight-year cumulative treatment costs for those cases did not differ significantly from that of patients who had tooth-supported restorations (Incici *et al.* 2009).

Patients with DD-I may likely be advised to have extraction and denture placement because of their poorly developed root structure and periapical radiolucencies (Seow 2003). Because of the short roots and possible mobility, routine orthodontic force should not be applied to these patients (Kalk *et al.* 1998).

Teeth in patients with DD-II and DGI-II that have adequate shape, size, and support can be restored with full coverage restorations if necessary. To improve esthetics, discolored anterior teeth may be treated with bleaching, resin bonding, veneering, or full coverage esthetic restorations. Teeth with favorable crown-to-root ratios, which minimize the risk of cervical fracture, have the best success with full coverage restorations. Pulpal therapy may be rendered in cases with detectable pulp canals and adequate radicular dentin structure, although the long-term prognosis for endodontically treated teeth is guarded (Rankow & Miller 1984).

Patients with DGI-III may suffer multiple pulp exposures in the primary dentition because of the shell teeth characteristic; however, they may not experience similar painful abscesses in the permanent dentition. Early treatment may reduce the episodes of painful pulpal exposures and the need for endodontic therapies. In severe DGI-III patients, treatment may be performed in two stages under sedation. Stage 1 treatment, performed at approximately age 18–20 months, is directed at protecting the incisors with bonding restorations and the first molars with preformed crowns. Stage 2 treatment, performed at approximately age 28–30 months, provides the second primary molars with preformed crowns and the canines with bonding restorations (Sapir & Shapira 2001). Both composite and fluoride-releasing glass

ionomer restorations have been used successfully, although due to the abnormally formed dentinoenamel junctions, bonding restorations may require frequent repair and replacement. Alternatively, overdentures can be placed during primary and mixed dentition stages with frequent adjustment or replacement to accommodate growth and to protect the remaining teeth (Walter 1988). Mouth guards can be fabricated to protect minimally affected dentitions and prevent further attrition.

Clinical course of a patient with dentinogenesis imperfecta type II

A 12-year, 9 month-old Caucasian female presented with amber brown discoloration of her teeth. She was healthy with no skeletal abnormalities or hearing disturbances. Her mother and brother also had discolored teeth, a heritable condition present in her family for at least four generations.

Clinically, the patient was in the late mixed dentition stage. Adequate attached gingival width, minimal bleeding on probing, and no sign of inflammation indicated that the patient's oral hygiene was acceptable. Radiographic examination (Figure 17.3A) revealed normal trabeculation, no loss of alveolar bone, and a permanent dentition with congenitally missing (1, 4, 13, 16, 29, and 31) or unerupted (2 and 15) teeth. The most striking features included bulbous crowns, thin roots, obliterated pulp chambers and root canals, and a decreased crown-to-root ratio in favor of the root length due to the small clinical crown and thin enamel.

A team made up of orthodontic, periodontic, and prosthodontic clinicians was assembled, and a comprehensive treatment plan was constructed with an overall goal of providing the patient with a functional and esthetically pleasing fixed reconstruction. The apparent challenges included limited retention, limited space, decreased vertical dimension, lengthy treatment duration, financial limitations, and the absence of an enthusiastic commitment from the patient and her family to engage in a routine maintenance program.

A diagnostic wax-up was performed to analyze the occlusal relationship, the size of the core buildups, and the fixed prostheses. The treatment plan included extracting the primary molars and closing the spaces with three-unit-bridges. The unrestorable maxillary molars (2, 3, 14, and 15) were to be extracted and replaced with a removable partial denture. All of the remaining teeth were to be fitted with single, porcelain fused-to-metal crowns. It was recommended that the patient wear an occlusal splint at night to protect the restorations.

Although the patient's mother had a similar dental defect resulting in extractions and complete dentures, she was hesitating to agree to the suggested comprehen-

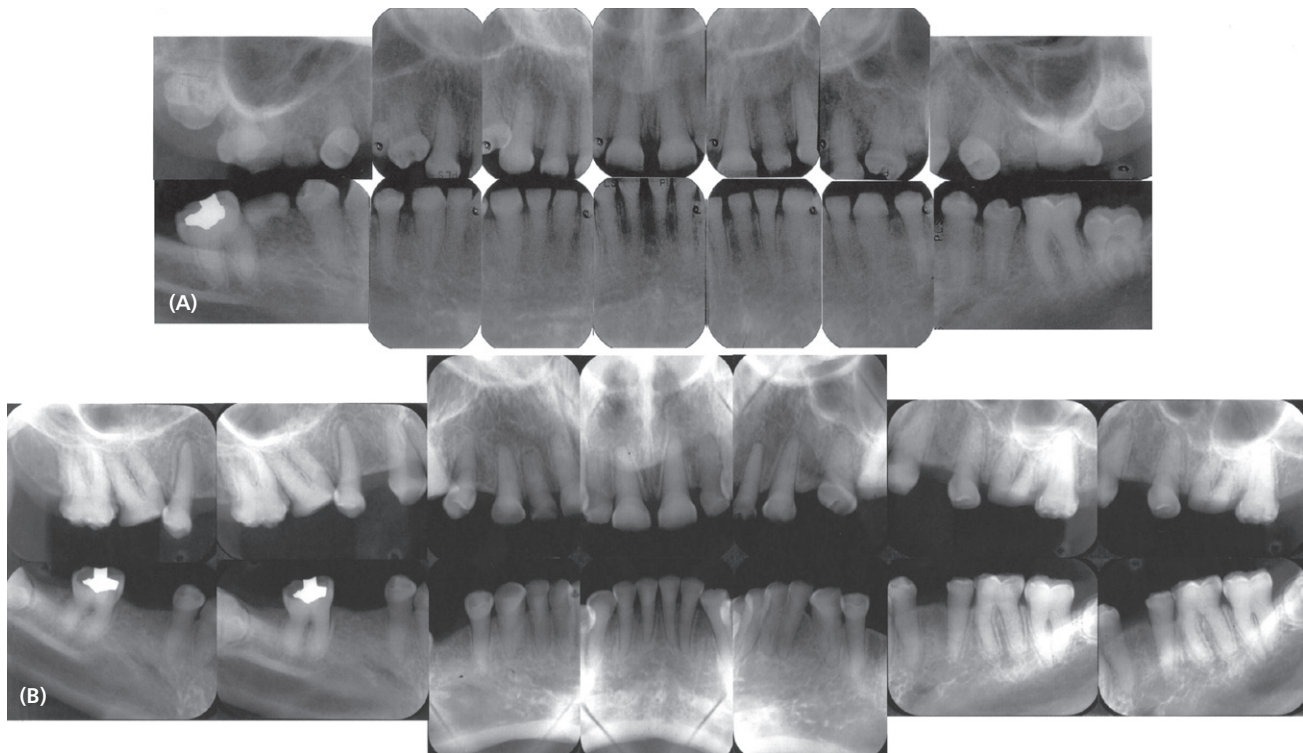


Figure 17.3 Radiographic records taken at the initial visit and post-orthodontic treatment. A. Full-mouth radiographs taken at age 12 years and 9 months revealed a mixed dentition with overretained primary molars A, J, and T and congenitally missing teeth 1, 4, 13, 16, 29, and 31. The teeth are generally small and their pulp chambers are obliterated. Among the incisors, the enamel appears to be thin and absent from incisal edges. B. Full-mouth radiographs taken post orthodontic treatment at age 17 years and 3 months.

sive treatment plan that would provide a better quality of life for her daughter. She was skeptical since she was traumatized at a young age by her own dental treatment consisting of extractions and dentures. The treatment planning and discussion took more than a year and was complicated by a long commute to the dental school clinic. The diagnostic wax-up was important in identifying the required steps for treatment and developing a strategy. Furthermore, it provided the patient and her family with a prospective view of the possible outcome of the reconstruction. After a thorough analysis and explanation, the patient's mother agreed to the suggested treatment plan for her daughter, who was then 14 years and 5 months old.

The extraction of teeth A, J, and T was completed in the urgent care phase. The corrective phase began with surgical crown lengthening to increase the clinical length of all crowns for attachment of brackets and retention of the restorations. In the posterior regions, osteoplasty was limited by short root trunks and concerns of exposing the furcation. During the maxillary surgery, the excessive labial frenum was removed to allow closure of the diastema orthodontically. The surgical procedures were performed under intravenous sedation and local

anesthesia and were completed in three appointments. The postoperative care included peridex rinses after meals and vicodin for pain control.

Following an uneventful healing period, orthodontic treatment was initiated to align the teeth. The maxillary premolars were derotated, the central diastema was closed, and the vertical overbite and occlusal plane were corrected (Figures 17.3B and 17.4). Treatment was carried out over a period of 36 months. In addition, a gingivectomy of teeth 19–27 was added to the treatment plan to increase the crown height. After reflection of undisplaced flaps, the root surfaces were degranulated and the flaps were positioned with sutures. Osseous surgery was not indicated as the biologic width between the crest of the alveolar bone and the intended crown margin was sufficient. The temporary restorations were cemented. The wound healing was uneventful, and after four weeks the physiologic tissue contour could be perceived (Figure 17.5). During the final preparation of all of the teeth, the crown margins were placed to follow the free gingival margin. The corrective phase of treatment was finalized with the permanent cementation of all crowns and bridges. After completion of treatment, the patient failed to return for follow-up maintenance care.

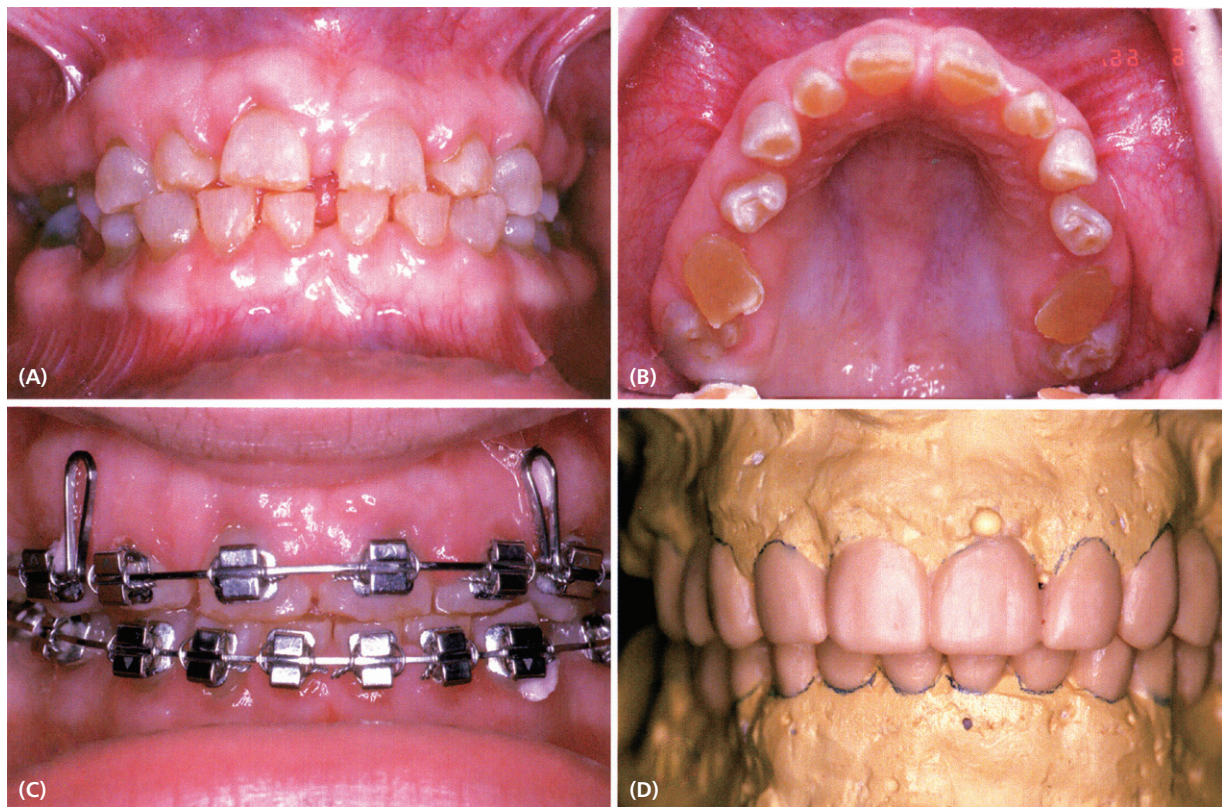


Figure 17.4 Photographs taken pretreatment and during the active treatment phase. A. Central diastema is evident in the maxilla and mandible. Teeth are yellowish to brownish. All incisal edges display signs of excessive attrition. B. The extent of the attrition in the first molar is evident. The dentin is exposed in load-bearing areas. C. Facial view taken during the orthodontic treatment phase. D. Post orthodontic therapy, study models were fabricated and mounted for analysis of the occlusion. The diagnostic wax-up facilitated treatment planning and communicating the treatment plan to the patient.

Eight years after the active treatment, the patient was located and urged to return for a follow-up visit. The patient reported that the reconstruction was esthetically pleasing and functionally excellent for a period of 3–4 years. Crown fracture of mandibular incisors and loss of porcelain facing on tooth 10 were later experienced. However, having no dental insurance coverage and a long distance of travel, the patient was not able to return for re-treatment and maintenance care.

Discussion

Current understanding of inherited dentin defects

Inherited dentin defects are a group of autosomal dominant disorders with abnormal dentin and pulp structure affecting both the primary and permanent dentitions. The commonly used classification scheme proposed by Shields has three subtypes of DGI and two subtypes of DD based on distinguishing clinical and radiographic

features. This classification scheme does not reflect the underlying genetic etiologies of these disorders. The genetic etiology of DD-I is unknown. DGI type I, which is associated with OI, is the result of mutations affecting the synthesis and processing of collagens leading to quantitative and/or qualitative defects. Current evidence supports the premise that all other subtypes of DGI and DD-II are consequences of mutations in the DSPP gene, which leads to an evolving concept that these conditions, while varying in severity, are disorders of a single genetic entity (Kim & Simmer 2007). As the genetic etiologies of inherited dentin defects became known, revising the classification scheme for hereditary dentin defects is becoming necessary (Dean *et al.* 1997).

Diagnosis of inherited dentin defects continues to be based on family history and clinical findings, since genetic diagnosis is available only in research laboratories (see, e.g., <http://ncbi.nlm.nih.gov/sites/GeneTests/lab?db=genetests>). Genetic testing by clinical laboratories on fee-for-service basis may become available and affordable if disease-causing mutations continue to be



Figure 17.5 Photographs showing the crown-lengthening procedure. A–B. The clinical crown was too short to support a casting crown. Therefore, gingivectomy and undisplaced flaps surgery were performed. Since the width of the attached gingiva was adequate, the initial incisions were made with a 1–2 mm scallop. C–D. After reflection of full thickness flaps, the roots were debrided and the flaps were sutured back into place. The clinical crowns gained length and accommodated apical positioning of the crown margins. E. Sutures were removed one week after surgery. F–G. The wound healing at four weeks after surgery was adequate, and the tissue contour followed the tooth alignment. The frontal view shows the temporary restorations in both jaws. H. Provisional restorations were delivered. Final restoration was completed at age 18 years and 4 months (photograph is not available).

identified and their associated phenotypes thoroughly characterized.

Despite extensive morphologic studies of the structure of teeth with dentin defects, the pathophysiology of this group of disorders is not understood. The characteristic dysplastic dentin with calcospherites and abnormal collagen composition provides no clue as to how odontoblasts are disturbed. The radicular or coronal location of dysplastic dentin used to distinguish DD-I and DD-II presents a perplexing issue: how could a seemingly normal area of dentin be produced in affected teeth (Melnick *et al.* 1977; Ranta *et al.* 1990)? Biochemical studies of DSP, DPP, and the characterization of DSPP-null mice have increasingly shed light on dentin mineralization. Mutation studies have revealed genotypic and phenotypic information that allows a better understanding of how defects in DSP and DPP may lead to dentin structural malformation. Mutations in the exon 5 region of DSPP between residue Asp⁵⁶² and Asp⁶⁸⁸ led to −1 frameshift and missense protein production resulted in DD-II, while a majority of the DPP mutations downstream to residue Asp⁶⁸⁸ resulted in a more severe DGI-II phenotype (McKnight *et al.* 2008; Nieminen *et al.* 2011).

Clinical management: limitations and possibilities

As inherited dentin defects affect both the primary and permanent dentitions to different degrees, dental management will vary depending on the severity of the effect. The primary goal of the treatment plan should be to address each concern as it presents with an overall comprehensive plan that outlines anticipated future treatment needs (American Academy of Pediatric Dentistry 2010–2011). For patients with DD-I, preventive care is critical. The goal of intervention is to retain the teeth for as long as possible to complement proper facial development. For individuals with DGI, optimal care includes preventing severe attrition associated with enamel loss and rapid wear of the poorly mineralized dentin, rehabilitating dentitions that have undergone severe wear, optimizing esthetics, and preventing caries and periodontal disease.

Genetic testing of individuals with DGI-I/OI can be performed in certified laboratories using 10 ml of peripheral blood sample from the test subject with a physician's order. However, genetic testing of individuals with other types of dentin defects remains possible only as part of a research laboratory investigation. Should genetic therapy for inherited dental defects become a reality, genetic testing will need to be conducted to determine the causative gene for accurate diagnosis in order to devise an effective therapy. It is essential to provide

nondiscriminatory healthcare policies that will assist individuals with inherited dental defects to receive the care that has proven to be critical in improving their quality of life.

Summary

Inherited dentin defects are frequently encountered genetic disorders with varying incidences of 1:8000 to 1:100,000 depending on the specific subtype of defect. While syndromic dentin disorder may have signs and symptoms involving bone, cartilage, cardiovascular structures, and so on, the nonsyndromic disorders affect tooth dentin only. Shields classified the dentin disorders into three types of dentinogenesis imperfecta (DGI) and two types of dentin dysplasia (DD). DGI type I is osteogenesis imperfecta (OI) with DGI. In most cases, OI with DGI is caused by mutations in the genes encoding type I collagen. However, OI is a collective term for a group of complex disorders with a broad spectrum of severities and genetic etiologies. DD-II, DGI-II, and DGI-III share many similar clinical and radiographic features, but each has its own unique presentation. They are caused by mutations in DSPP, which encodes the major noncollagenous proteins of the dentin matrix. The etiology remains unknown for DD-I, a rare condition having an incidence of 1:100,000 and featuring short, blunt roots and obliterated pulp chambers. The abnormal matrix deposition, mineralization, and odontoblast turnover in inherited dentin defects result in unsound dentin/enamel junction, fragile enamel, and soft dentin that becomes severely compromised upon occlusion. Significant attrition and discoloration of the primary and permanent dentitions in dentin disorders necessitate dental rehabilitation at an early age. Retreatment is often necessary during adulthood, and the long-term prognosis is guarded. This chapter presents the clinical course and management of patients with nonsyndromic hereditary defects of tooth dentin in the primary and early permanent dentitions.

Acknowledgments

We dedicate this chapter to the families whose participation in genetic studies has advanced our knowledge in the etiology and management of inherited dental defects.

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Enamel fabrication: the story of amelogenesis

Carolyn W. Gibson and Malcolm L. Snead

Development of teeth and gene expression

Developing teeth have been used to identify the reciprocal signals that serve to determine organ morphogenesis and terminal cell differentiation. Deciphering the signaling pathways responsible for establishing dental identity has been achieved with elegant genetic experiments based upon gain of function, loss of function, and transgenic experimental strategies with members of gene families and their receptors. However, these efforts have focused almost exclusively on signals for cell determination and identity, events occurring during early tooth development. They have not focused on the outcomes from such signals that occur during later development stages, such as the control over enamel gene expression that is needed to produce a biomineralized ceramic tissue that defines a “tooth.” Nonetheless, modest progress has been made toward understanding gene expression regulation for the ameloblastin and amelogenin genes (Dhamija *et al.* 1999). At the start of enamel matrix formation, an increase in the transcription factors CAAT/enhancer binding protein- α (C/EBP- α) drives the ameloblasts out of the cell cycle, an action that is opposed by Msx2 (Zhou *et al.* 2000). C/EBP- α also upregulates the expression of enamel matrix proteins, such as amelogenin, ameloblastin, and other structural proteins (Xu *et al.* 2007). Along with synthesis, the ameloblasts are simultaneously responsible for proteolytic processing and re-absorbing degraded proteins during mineral replacement (Li *et al.* 2001; Caterina *et al.* 2002; Simmer & Hu 2002; Shapiro *et al.* 2007). While not yet identified in an affected individual, a change to the enamel phenotype is expected to be possible with alterations to any of these genes that serve to regulate (1)

the abundance of proteins, (2) critical posttranslational modification, (3) a structural role in the enamel matrix, (4) a role in cell adhesion, and/or (5) degradation of proteins. A role for microRNAs (miRNAs) in altering any of these critical pathways has also been recently identified, although no known examples of enamel malformations have yet to be reported for miRNAs (Michon *et al.* 2010).

Enamel matrix proteins and proteinases

Enamel is the remarkable bioceramic composite covering the vertebrate tooth. Oral ectoderm cells differentiate into ameloblast cells to synthesize the protein mixture that assembles to form the extracellular enamel matrix that is the active interface that guides mineral crystallite habit and orientation. Matrix formation must occur properly for the proteins to guide their own replacement by the carbonated hydroxyapatite mineral phase. There are no second chances to correct enamel matrix production defects since enamel does not remodel, and with tooth eruption, the ameloblasts cease to exist. Making enamel is a one-time exercise that must be done without errors.

The enamel extracellular matrix is composed principally of amelogenin protein, with the second most abundant protein being ameloblastin (Fincham *et al.* 1999). While other “structural” enamel proteins exist, they are at reduced abundance compared to amelogenin or ameloblastin. These proteins members contribute to the assembly of the enamel matrix and include enamelin (Hu *et al.* 2005, 2008), tuftelin (Deutsch *et al.* 1991), amelotin (Iwasaki *et al.* 2005; Moffatt *et al.* 2006), and transient sulfated proteins (Smith *et al.* 1995). A precise

role for each of these proteins during enamel formation is not yet well defined. In addition, a variety of proteinases that play a significant role in removal of the enamel extracellular matrix proteins have been identified (Simmer & Hu 2002; Bartlett *et al.* 2004; Simmer & Bartlett 2004). These proteins include highly specific proteinases that can degrade amelogenin and ameloblastin. They also include “less specific” proteinases that serve to further degrade the enamel matrix proteins so that they can be removed by the very ameloblasts that previously synthesized them. When enamel formation is finished, the enamel contains less than 0.5% protein (Smith 1998).

This process of biosynthesis and degradation by a single cellular entity assures coordination between the anabolic and catabolic phases of development (Figure 18.1). Equally noteworthy is the fact that degradation of enamel matrix proteins appears to occur simultaneous with assembly. However, degradation is accelerated during the mineral maturation phase in which crystallites grow in girth, becoming closely packed one to another and filling the space previously occupied by the matrix (Smith *et al.* 2005). While some residual proteins remain, they are but a tiny fraction of what was once there. Attention has been focused of late on the role that these residual proteins serve to modify the behavior of the carbonated hydroxyapatite, altering its mechanical properties and resulting in improvements to the mech-

anical properties of the final enamel bioceramic (White *et al.* 2001).

Ameloblast cell biology, integrins, and cell adhesion molecules

Prior to enamel matrix production, pre-ameloblasts sit opposite the neural-crest derived odontoblasts, separated from them by the basement membrane. Ameloblasts synthesize and secrete the initial enamel matrix into the space previously occupied by the basement membrane (Brownell & Slavkin 1980). The area receiving the first wave of enamel matrix proteins is the future dentine-to-enamel junction (DEJ or EDJ, as one prefers); this is a “specialized” enamel that links these two dissimilar bioceramic tissues, enamel and dentine, to one another (Fong *et al.* 2003; White *et al.* 2005).

During biosynthesis, registration of ameloblast to a field of enamel matrix results from the sum of at least two categories of interactions. The integrins (Salmivirta *et al.* 1996) and CD63/LAMP (Tompkins *et al.* 2005; Bartlett *et al.* 2006) are the well-known receptors located on the ameloblast secretory termini (Tomes’ processes) that recognize amino acid motifs within enamel matrix proteins. Additional interactions occur through the actions of lectin-like regions within the secreted amelogenin that bind N-acetyl glucosamine residues on membrane proteins embedded on the secretory surface of

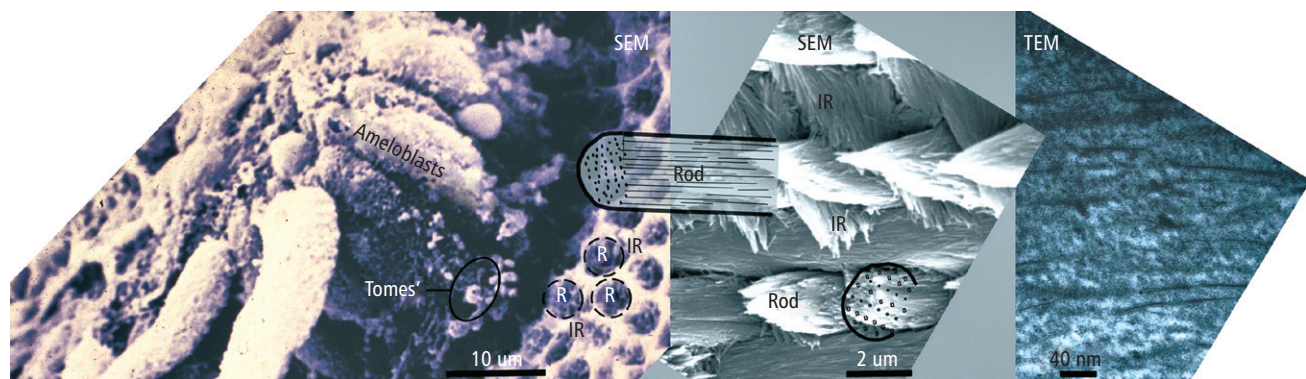


Figure 18.1 Synoptic cartoon of enamel formation. Ameloblast cells (left panel) exert hierarchical control over each step of amelogenesis, synthesizing the proteins and secreting them via Tomes’ processes. The ameloblasts stay in registry with their respective field of enamel matrix proteins via receptors and lectin-like interactions between the ameloblast and the matrix proteins. Each ameloblast organizes one rod (R) of enamel matrix proteins, and each rod will eventually consist of thousands of crystallites of carbonated hydroxyapatite. The “C”-shaped boundary of the rod permits the formation of the interrod (IR) enamel matrix (middle panel) that is eventually converted to mineral. A transmission electron micrograph (TEM) image of negative-stained enamel matrix that consists of a complex mixture of proteins, as described in the text, is seen in the right panel. The dominant protein, amelogenin, self-assembles to form nanospheres seen as electron lucent nanospheres surrounding HAP crystallites (black stained ribbons). With degradation, ameloblastin forms a “sheath” defining the lateral boundaries of each rod. This unique organization of the crystallites yields a bioceramic composite material with unique wear properties that allow it to last a lifetime. The wear-resistant but brittle enamel is coupled to the softer but tougher underlying dentine via the DEJ forming a composite system that is made by living cells and not a chemist or engineer.

ameloblasts (Ravindranath *et al.* 1999). When disruption to these two pathways reaches a critical level, such as in the presence of a defective matrix due to mutations in matrix protein domains, the ameloblasts can lose contact with the matrix and re-enter the cell cycle (Zhu *et al.* 2006; Fukumoto *et al.* 2005).

The complicated and uncorrectable production steps for enamel formation require the ameloblasts to remain precisely orchestrated with respect to a specific segment of the forming matrix. Cell-to-matrix and matrix-to-cell interactions are accomplished by linking one ameloblast to one enamel prism rod, the basic unit of enamel (Figures 18.1 and 18.2; Boyde 1969). The cell must stay in contact with its cylinder of matrix proteins during a migration of thousands of micrometers during matrix production (Osborn 1970). Ameloblasts interact with the forming enamel matrix via receptors embedded in the Tomes' processes of ameloblasts. Evidence suggests that Tomes' processes contain members of the integrin family (Salmivirta *et al.* 1996; Fukumoto & Yamada 2005), including $\alpha 6$, $\beta 2$, and $\beta 4$. These integrins are candidate molecules that could be used to maintain the registration of the ameloblast over the forming enamel matrix. Investigators have recently shown that amelo-

blasts can interact with an artificial RGD domain found on nanofabricated peptide amphiphiles (RGD-PA) (Huang *et al.* 2008). Here, primary enamel organ epithelial cells from developing mouse teeth can be embedded within a gel of RGD-PAs where we observed their enhanced proliferation, followed by their differentiation with increased expression of mRNAs encoding enamel matrix proteins. Similar changes to gene expression are observed among enamel organ epithelial cells at the site where RGD-PA is microinjected into developing incisors (Huang *et al.* 2008). We have determined that ameloblast cells grown in the RGD-PA upregulate their expression of $\alpha 6$ -, $\alpha 5$ -, and $\beta 4$ -integrins and that these integrins are present but at lower expression levels in the untreated controls' organs. These data support the notion that integrin-mediated interactions between the ameloblast and the matrix occur during enamel formation (Salmivirta *et al.* 1996; Fukumoto & Yamada 2005).

Data from Ryan and colleagues (1999) also support the notion that the integrin $\alpha 6$ expression by secretory ameloblasts is essential to enamel formation. In their laminin-defective animals, they observed that ameloblasts fail precisely during the secretory stage of development. Quoting from their publication, "In mutant

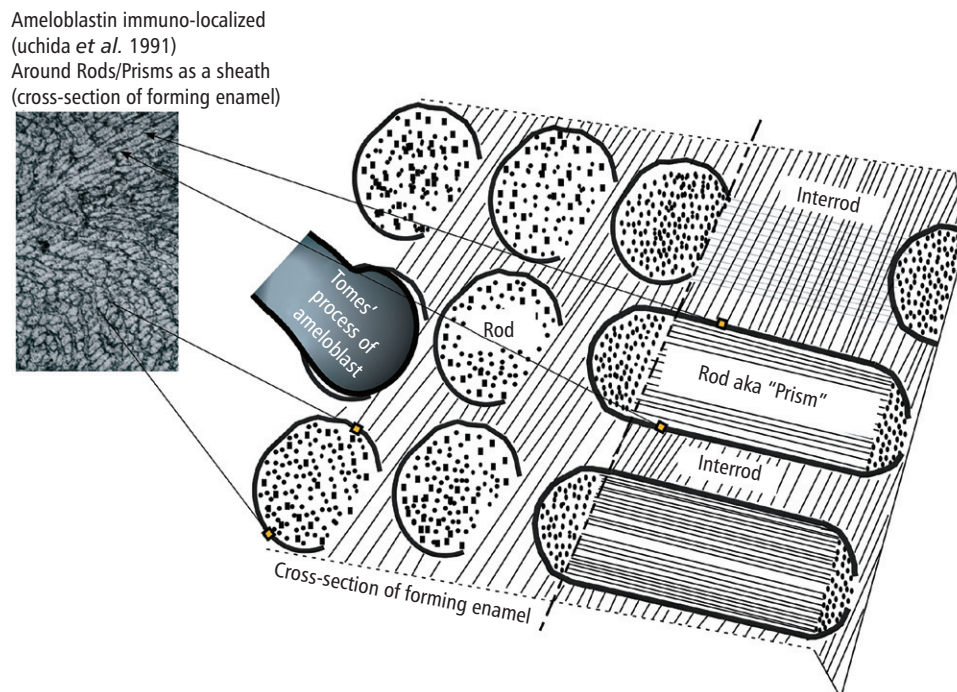


Figure 18.2 Cartoon of newly formed enamel matrix and crystallites. Patterning the rod-to-interrod boundaries is the hypothesized function of ameloblastin (i.e., sheathelin). The forming enamel is immunostained by an antibody against ameloblastin forming a honey-combed pattern of the rod boundaries. These correspond to the solid dark line surrounding the rods in the cartoon (Uchida *et al.* 1991). Tomes' processes secrete proteins into the extracellular space, becoming embedded in the forming matrix that corresponds to a one-enamel rod. Tomes' processes interact with the forming matrix during all phases of its creation and are responsible for the final geometry that contributes to the unique mechanical properties of enamel.

animals, incisor development appeared normal until the onset of enamel secretion” (Ryan *et al.* 1999, p. 1318). Moreover, the observations of Wright and colleagues on the dentition of patients affected with epidermolysis bullosa similarly support the requirement for cell-to-matrix interactions during enamel formation (Wright *et al.* 1996).

In addition to integrins, several other proteins have been nominated as candidates for amelogenin receptors, including CD63 and LAMP1 (Tompkins *et al.* 2005). Both CD63 and LAMP1 are located on Tomes’ process in direct contact with the forming enamel matrix. CD63 is a member of the tetraspanin family characterized by the presence of four transmembrane segments (Stipp *et al.* 2003). The tetraspanin proteins mediate signal transduction events that play roles in the regulation of cell development, activation, growth, and motility (Yunta *et al.* 2003). CD63 and other tetraspanins are known to complex with integrins and act as organizers of membrane microdomains and signaling complexes (Berdichevski 2001; Yunta *et al.* 2003). LAMP1 is a transmembrane protein highly expressed in late endosomes and lysosomes and is often used as a marker for these two organelles (Cook *et al.* 2004). LAMP1 immunoreactivity is observed at the plasma membrane of ameloblasts and of organelles in the early endocytotic compartments (Shapiro *et al.* 2007).

Hierarchical organization and material properties

Comparison of enamel formation and its resulting material properties between mammals is in its infancy. There have been but a few examples of prospective efforts to redesign enamel formation by altering the function of enamel matrix proteins, and these have been limited to engineering the amelogenin protein (Zhu *et al.*, 2004, 2006). Similarly, there have been but a few examples of efforts to redesign the dentine enamel junction by over-expressing proteins believed to contribute the favorable mechanical properties of this unique union (White *et al.* 2000, 2007). However, recent work has also shown the importance of gradients of material properties to influence the deformation of the dentine at the DEJ (Zaslansky *et al.* 2006).

For perspective, until very recently there was debate regarding the number of genes and proteins that contribute to enamel formation. Sequencing the genomes of several model organisms, including mouse and human, has contributed to our understanding of the genomes and their contribution to amelogenesis. However, the protein-to-protein interactions that define matrix assembly as well as the disassembly of the matrix by

various proteinases had only just begun to be explored (Paine & Snead 1997). What has greatly advanced this field of study has been molecular dissection of the gene mutations that affect the function of enamel matrix proteins. The old adage that “one mutation is worth a thousand biochemists” comes readily to mind. From the analysis of such mutations, previously unrecognized enamel defects have begun to be mapped to mutations in the domains of enamel protein that serve to regulate the physiologic function of that protein. This is illustrated in the example of a mutation in the amelogenin gene in a family that results in remarkable changes to the enamel, supporting a role for enamel in cell-to-protein interactions and in the production of a matrix that guides its own replacement to the mineral. However, there are all too few examples of informative studies being performed in humans for a variety of ethical and logistic reasons. Therefore, most experiments are performed in mice, and we presume that the lessons learned from rodents will largely apply to the human condition. Of mice and men continues to be a quandary for many basic and translational scientists.

The terms *prism* and *rod* are used here to mean the bundle of hydroxyapatite (HAP) crystallites formed from the enamel matrix secreted by a single ameloblast cell. The term *interrod* or *interprism* is used to name the matrix between these boundaries and that gives rise to mineral continuity within enamel (Figure 18.1). The most abundant protein of the forming-enamel matrix, amelogenin, undergoes self-assembly to form nanospheres and spheres that, by definition, are fully symmetrical. It is the amelogenin nanospheres that serve to guide formation of the long- and thin-crystallite habit, regardless of their relative positions to the ameloblast. Nonetheless, the forming-enamel matrix is “patterned,” and it is the distribution of ameloblastin that serves to segregate the matrix into rod or interrod regions.

The ameloblast first creates a 100% organic enamel extracellular matrix that initiates the enamel formation process. Ultimately, this matrix is converted to 99.5% inorganic hydroxyapatite crystals (Simmer & Fincham 1995; Robinson *et al.* 1998; Smith 1998; Fincham *et al.* 1999). Enamel formation is studied using genetic engineering techniques in order to control the proteins that contribute to matrix assembly. Since matrix assembly must first occur properly for the enamel–mineral phase to be subsequently created, this approach allows us to modify the final enamel bioceramic during its production phase. Understanding matrix formation is essential, therefore, to understanding the mineral phase. In newly synthesized enamel matrix, immunodetection techniques identify extracellular ameloblastin protein dis-

tributed randomly across the width (cell diameter) of the protein matrix cylinder that constitutes an enamel rod. With time, proteolytic cleavage and mineral replacement of the matrix occurs, and the ameloblastin protein redistributes to the circumferential boundaries of the cylinder (Figure 18.2), outlining the rod boundaries, except on the inferior surface where two rods coalesce into the interrod. It is this linkage between the cell interacting with the matrix and the matrix controlling the mineral habit and packing that accounts for the unique physical properties of enamel.

Nanci and colleagues (1998) found that mineral deposition lags slightly behind the site of secretion at the Tomes' process. In this mineral-free zone, enamel matrix proteins undergo assembly into a matrix that is competent to guide the mineral phase crystallite habit as long thin crystallites aligned along their *c'*-axis (long crystallographic axis) parallel to a path that the ameloblast has moved, a process defining the rod. The portion of Tomes' process that is confined to the pit defines where the enamel rod will form (Figure 18.1). Due to the close abutment of ameloblasts to their neighbors, the Tomes' process from multiple neighbors can make a contribution to the interrod matrix, allowing it to accumulate faster than the rod, resulting in the apparent "dimple" in the forming matrix when the ameloblast is mechanically removed (Figure 18.1). At this developmental stage, the matrix is a hydrated gel with nucleating sites dispersed within it. The ameloblasts "hover" over this protein gel, with the Tomes' process embedded in the gel. Integrins, located in the ameloblast membrane, are believed to guide cell-to-matrix interactions by interacting with ameloblastin protein domains (Fukumoto *et al.* 2004, 2005).

Enamel architecture reflects its dependence upon stereotactic control during its formation where even minor changes to matrix formation become permanently recorded in the resulting mineralized enamel. The ameloblast cell secretes the enamel matrix through Tomes' process. One ameloblast cell is responsible for the formation of one enamel rod and its adjacent interrod matrix. An ameloblast cell secretes and then interacts with a cylinder of enamel matrix, approximately the same diameter as the ameloblast, starting at the dentine and extending outward toward the chewing surface of the tooth. Within each rod, thousands of substituted hydroxyapatite (HAP) crystallites form, first as thin crystallites surrounded by amelogenin nanospheres that promote growth along the crystal ends. The basic design of enamel revolves around the organization of these enamel matrix rods, one to the other, with the ameloblasts weaving the cylinders of enamel matrix proteins so that they coalesce on their inferior surface, produc-

ing a linkage between each rod, termed the *enamel interrod* (Figure 18.1). The unique material properties of the final mineralized enamel are dependent upon the weaving of rods and upon the coalescence among rod boundaries at the interrod (Paine *et al.* 2000). Coalescence between rods and the interrod results in a continuum among crystallites yielding the unique material properties of mature enamel that allow it to last a lifetime of wear (White *et al.* 2001; Fong *et al.* 2003; Snead *et al.* 2006). Enamel matrix proteinases soon degrade the amelogenin-rich matrix while the crystallites grow in thickness, eventually lying "cheek to jowl" next to one another in the space previously occupied by the proteins (DenBesten & Heffernan 1989; Smith 1998; Simmer & Hu 2002; Bartlett *et al.* 2004). Immunohistochemical evidence shows that ameloblastin is proteolytically processed into an N'- or a C'-fragment (Kobayashi *et al.* 2007) and that each of these fragments localize within the rod with a distinctive distribution. This unique spatial distribution suggests that ameloblastin may control the relationship of the rods to one another and thus control the material properties of the resulting bioceramic composite (Uchida *et al.* 1991; Hu *et al.* 1997).

Investigators in recent years have shown that enamel can be thought of as tissue that represents a tough fibrous continuum rather than separated cellular automata and that this continuum contains discontinuities (Warschawsky & Smith 1971; Boyde 1987; White *et al.* 2001). Remnants of the enamel extracellular protein matrix create these discontinuities. Errors in matrix assembly can occur by spontaneous mutation, as can occur in amelogenesis imperfecta (AI), or the error can occur by genetic redesign of the domain(s) of proteins that contribute to the matrix. Such errors usually result in a matrix that serves poorly to guide the formation of the mineral phase. In these instances, the matrix errors are consequently recorded in the form of additional discontinuities that alter the material properties of the resulting enamel. However, far from being solely a destructive influence, these discontinuities can also improve the toughness of the biologically fabricated HAP, making it tougher than geological HAP (White *et al.* 2001). Fracture mechanics has provided an important method useful in producing quantitative measurements of changes to the formed enamel. Coupled with nanomechanical testing (Fong *et al.* 2003), these techniques produce damage at several scale features and serve as a powerful tool to interrogate the organization of enamel structure. The altered enamel structure can be created by inherited mutations as has occurred for the family members that are affected by an enamelin mutation. For review, see Chapter 19 in this volume.

Disruption to the enamel extracellular matrix due to gene mutation

The inherited enamel defect caused by gene alteration is termed amelogenesis imperfecta (AI)

Mutations in different genes lead to different AI phenotypes and patterns of inheritance. Witkop and Sauk (1976) described three general categories:

- Hypoplastic AI, in which the enamel layer is abnormally thin
- Hypocalcified AI, in which there is a defect in the mineral crystals
- Hypomaturational AI, in which proteins are insufficiently removed, leaving enamel that is soft and discolored

Many subcategories have also been reported, and interest has developed in linking gene mutation to phenotype for more effective treatment planning. Because AI is a relatively rare condition (1:700 and 1:14,000 in two reports), understanding the inheritance pattern can help distinguish genetic mutations from other causes of defective enamel. For a review, see Ng and Messer (2009).

Gene mutations that lead to AI include:

- Amelogenin (AMELX) is the most abundant enamel protein and regulates thickness and structure of enamel.
- Enamelin (ENAM) encodes a 120kD protein precursor that is cleaved to generate an active 32kD component. (See the accompanying clinical correlate in Chapter 19, this volume.)
- Matrix metalloproteinase 20 (MMP20) is a protease that begins the processing of enamel proteins during secretion stage. Lacking a normal gene leads to hypomaturational, as too much organic material remains upon tooth eruption.
- Kallikrein 4 (KLK4) encodes a protease that removes enamel proteins that still remain during enamel development. Mutation also leads to hypomaturational, which cannot be alleviated later during development, as the ameloblast cells are lost by the time the tooth erupts into the oral cavity.
- FAM83H encodes an intracellular ameloblast protein associated with internal vesicles and a variable phenotype (Ding *et al.* 2009; Wright *et al.* 2009).
- WD repeat domain 72 (WDR72) encodes a putative beta propeller considered a scaffold for proteins to interact intracellularly (El-Sayed *et al.* 2009).

The AIs have been considered nonsyndromic or affecting only enamel; yet many of the listed genes have low levels of expression in other tissues, without obvious

additional phenotype in AI patients. The gene mutation has not yet been identified in many additional AI kindreds, and it is assumed that additional gene mutations will be discovered in future work.

Gene defects have also been identified that lead to syndromes that include enamel defects; selected genes are listed below. For review, see Bailleul-Forestier *et al.* (2008).

- DLX3 is associated with trico-dento-osseous (TDO) syndrome. This autosomal dominant disorder affects enamel, hair, and bone (Price *et al.* 1998).
- CNM4 mutations lead to Jalili syndrome, which includes enamel defects and cone-rod dystrophy of the eye (Parry *et al.* 2009; Polok *et al.* 2009).
- Epidermolysis bullosa (EB) spectrum is due to a number of gene mutations and is characterized by blistering. It is sometimes associated with enamel defects (Wright 2010a).

Use of mouse models to better understand enamel defects

Kindred analysis for identification of mutations can provide information about phenotypic variability and pattern of inheritance, but murine models have been developed in which any gene can be altered to understand better the function of the encoded protein. Murine teeth are similar to human teeth at the level of gene structure, DNA sequence and gene expression, and the developmental program that the enamel secreting ameloblast undergoes in these species is comparable. In addition, genetic alteration of mice is relatively easy to perform. Approaches have included *transgenic mice* in which a normal or mutated enamel protein gene is expressed in addition to the full complement of normal (wild-type or WT) gene expression, *null-* or *knockout* mice that have a mutation in a single gene that prevents expression of a normal protein, and *knock-in* mice in which a normal gene is replaced with a mutated one. In addition, genetically altered mice can be mated together to generate compound phenotypes. The advantage of this approach is that mice can duplicate the genetic changes in human AI patients to provide a window to follow development of AI and to provide ample teeth for evaluation.

Null mice have been described for *Amelx* (Gibson *et al.* 2001); *Ambn* (Fukumoto *et al.* 2004), later shown to express a truncated AMBN protein (Wazen *et al.* 2009); *Enam* (Hu *et al.* 2008); *Mmp20* (Caterina *et al.* 2002); and *Klk4* (Simmer *et al.* 2009).

The murine phenotypes are similar, in general, to those seen in humans with a similar mutation. These AI models have provided teeth for detailed analysis of the

enamel mineral and organic matrices leading to enhanced understanding of protein function. The mouse *Ambn* mutation has provided valuable functional information as a human *AMBN* mutation has not yet been reported.

Human gene mutations are frequently not null, but are subtle changes with alteration of but a single or a few amino acids. These mutations can also be generated in mice by knock-in experiments in which a mutated gene replaces the endogenous gene (Zhu *et al.* 2004; Simmer *et al.* 2009). Similarly, a null mouse can be mated with a transgenic mouse that has a constructed transgene with a defined mutation. In this way, any nonlethal combination can be produced allowing detailed analyses of the phenotype. Using these methods, one can learn how each enamel protein contributes to the complex prismatic structure of mature enamel and perhaps develop ideal treatments for the various types of AI.

Enamel defects caused by environmental influences

The ameloblast cells that produce enamel are uniquely sensitive to their environment. For example, fever can cause abnormal enamel structures to appear as horizontal ridges, and elevated level of dietary fluoride can lead to opaque enamel with brown patches and structural defects while tetracycline administration can produce discoloration. These agents have an effect only on unerupted teeth since the ameloblasts are no longer present after eruption and enamel formation is complete.

Caries can affect teeth at any age due to bacterial colonization of tooth surfaces and degradation of the enamel layer. Caries is the most prevalent infectious disease of mankind (Caufield 2005). It is inhibited by drinking fluoridated water during dental development, which permits the fluoride ion to be incorporated into the crystal lattice of the HAP rendering it less soluble. The problem persists in regions of the world where access to fluoride is limited and in individuals with poor dental hygiene. Because ameloblasts are not present on the surfaces of erupted teeth, biological repair is not possible, and clinical intervention is required to alleviate carious lesions. A recent editorial describes potential genetic contributions to caries risk or protection (Wright 2010b).

Conclusions, future directions, and perspectives

Errors during enamel formation can result in clearly identifiable dental abnormalities. Clearly identifiable malformations of the teeth have also helped us identify protein domains among the enamel matrix proteins that are required for proper enamel formation. Recently, sci-

entists have been able to reverse engineer the mouse model organism creating phenocopies of human enamel disease states such as AI, epidermolysis bullosa, cystic fibrosis, and others diseases not primarily thought of as “dental.” This phenomenal advance has been made possible through transgenic or homologous recombination techniques that result in modification to the mouse genome and hence the phenotype. Of equal importance to these powerful prospective genetic tools for studies of animal models has been the completion of the human genome allowing studies of ourselves. As the reader surmises, all of the complicated elements of development and homeostasis apparent in the formation of other organs also operate during formation of the tooth. Therefore, one can anticipate that changes in cellular uptake through lysosomal pathways or changes to the function of solute channels that regulate ions and pH, and many other fundamental cell mechanisms, are also likely to have an impact on normal tooth development. When these pathways are altered by genetic mutations, we anticipate that they will exhibit a dental phenotype as well as affect other tissues in which their function is critical. In this way, the craniofacial apparatus is serving to remind us that the complexities of cell function are not excluded from enamel formation. Even though enamel erupts into the mouth as a cell-free tissue, it is entirely dependent on the complex cell functions that serve other tissues in our bodies.

Summary

Dental enamel is a bioceramic composite generated by ameloblast cells while a tooth develops within the mandible or maxilla. This unique, highly mineralized material is designed to resist the forces of mastication during the entire life of an individual without benefit of repair, as the cells that produce it are not present after the tooth erupts. During development, ameloblasts adhere to the secreted enamel organic and mineralized matrix by interactions between ameloblast cell surface proteins and the matrix. These interactions occur during the entire series of developmental stages that define enamel secretion and replacement of the secreted assembled proteins with mineral crystals organized into enamel rods that extend from the dental enamel junction (DEJ) to the enamel surface. The principal genes responsible for development of enamel have been studied, as have their mutations that lead to the errors of enamel formation known as amelogenesis imperfecta. Murine models have been generated to mimic the human condition in order to better understand the complex development of enamel and the function of the individual proteins during amelogenesis.

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Clinical correlate: amelogenesis imperfecta

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Amelogenesis imperfecta represents a group of hereditary conditions of the teeth characterized by marked genetic heterogeneity affecting amelogenesis in the primary and permanent dentitions and resulting in alterations to the organization and appearance of the enamel upon clinical examination. At the beginning of the formation of dental enamel, ameloblast cells within the dental organ secrete an organic matrix that immediately begins to mineralize. The major enamel protein components of this matrix include the most abundant amelogenin proteins as well as less plentiful proteins such as enamelin (Robinson *et al.* 1998). Mutations in any one of the genes encoding these and other proteins can lead to the defects in enamel formation that are collectively known as *amelogenesis imperfecta* (AI; Simmer & Hu 2001; Hu & Yamakoshi 2003; Wright 2006).

With advances in genetic analysis, the spectrum of AI disease has been resolved by identifying the affected gene. At the molecular level, there are many pathways that can account for altered protein function. Despite the wide range of genetic defects in AI, they all generally result in an enamel that is clinically observed to be too thin (hypoplastic) or too soft due to abnormal mineralization (hypocalcification or hypomaturational; Witkop 1976, 1988). The prevalence of AI varies according to the geographic area studied and ranges from 1:700 to 1:14,000. AI has been classified according to both the appearance of the enamel and the pattern of genetic inheritance (Witkop 1976, 1988; Aldred & Crawford 1995; Aldred *et al.* 2003; Wright 2006). As sufficient information is learned about the genetic causes of AI, a gene-based classification system will most likely be adopted.

The AI trait caused by mutations in the enamelin (ENAM) gene is often transmitted through an autosomal dominant mode of inheritance. While the specific role of enamelin in amelogenesis is unknown, it is thought to play a role in crystallite growth regulation and elongation. ENAM mutations generally result in hypoplastic AI, in which the hypoplastic teeth can exhibit a reduced clinical crown size with proximal space at the contacts due to reduced enamel thickness. Depending on the specific ENAM mutation, the hypoplastic enamel can be localized as pits or horizontal ridges or can be generalized with the entire enamel thickness being markedly reduced. In addition, several of the cases of ENAM gene mutations resulting in AI report an anterior open-bite malocclusion (Kida *et al.* 2002; Hart *et al.* 2003; Kim *et al.* 2005). Crown resorption of unerupted teeth and delayed eruption occur more frequently in AI patients than non-AI patients, but these have not been specifically linked to ENAM mutations. The defects of AI have aesthetic and psychosocial implications requiring extensive treatment of primary and permanent dentitions, as well as counseling (Coffield *et al.* 2005).

Case presentation

A 9-year, 11-month-old male was referred to the Children's Hospital of Philadelphia for dental evaluation (Lindemeyer *et al.* 2010). His chief complaint was that his permanent posterior molars were not erupting. His prenatal and medical history was noncontributory. His mother reported a normal pregnancy and delivery with no complications. The patient's father indicated a family history significant for AI; he had a cousin and a niece

who had been diagnosed with AI by their dentists. The patient's mother reported no history of AI.

Clinical examination of the affected individual revealed a mixed dentition. The patient reported no sensitivity of his teeth to thermal stimuli, difficulty eating, or pain. The enamel surface of the teeth appeared smooth; the teeth lacked proximal contacts and were generally yellow, a color consistent with the findings



Figure 19.1 Clinical presentation of a 9-year, 11-month-old male patient with autosomal hypoplastic AI. Teeth are smooth and lacking proximal contacts and have a generalized yellow color.

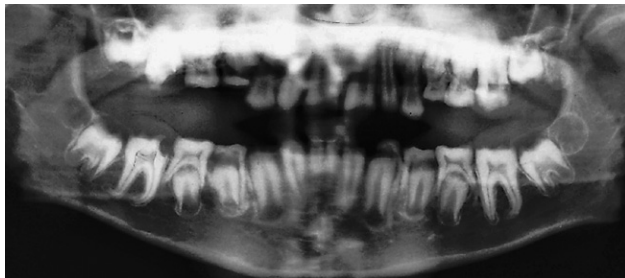


Figure 19.2 Panoramic radiograph of the patient. Note the unerupted permanent molars, with the maxillary first molars appearing externally resorbed.

of hypoplastic AI. There was no clinical evidence of pitting, grooves, or other irregularities in the enamel (Figure 19.1).

All of the first permanent molars were unerupted despite having radiographically visible complete root formation (Figure 19.2). The radiographic appearance of the teeth indicated thin enamel. The occlusal tables of the primary maxillary posterior teeth were observed to be well below the remaining occlusal plane, suggesting that these teeth were ankylosed. This may have been due to the hyperplastic gingival tissue and uneven resorption of some of the primary roots. The primary mandibular central incisors were present, and the permanent mandibular central incisors appeared to be congenitally missing. Soft tissues overlying the affected teeth were intact, and no periodontal pockets were detected on any adjacent teeth. There was enlargement of the gingival tissues in the molar region. The patient's occlusion appeared to be a bilateral Class I with an overbite of 100% (no anterior open bite) and an overjet of 2 mm. Radiographically, the unerupted permanent maxillary first molars appeared malformed with resorption of a portion of the clinical crowns (Figure 19.2); the permanent mandibular right first molar appeared to have a marked area of resorption on its mesial surface. There was no evidence of taurodontism.

After consulting with an oral surgeon and an orthodontist, a treatment plan was formulated to surgically expose the four permanent molars and extract all remaining primary molars. Saliva samples were collected from the patient and his parents for DNA analysis. The patient underwent oral rehabilitation under general anesthesia that included surgical exposure of the permanent first molars. The two permanent maxillary molars appeared to have deformed dentin, direct contact with the pulp chambers in both teeth, and little or no enamel (Figure 19.3). The teeth were nonrestorable, and a decision was made to extract them.

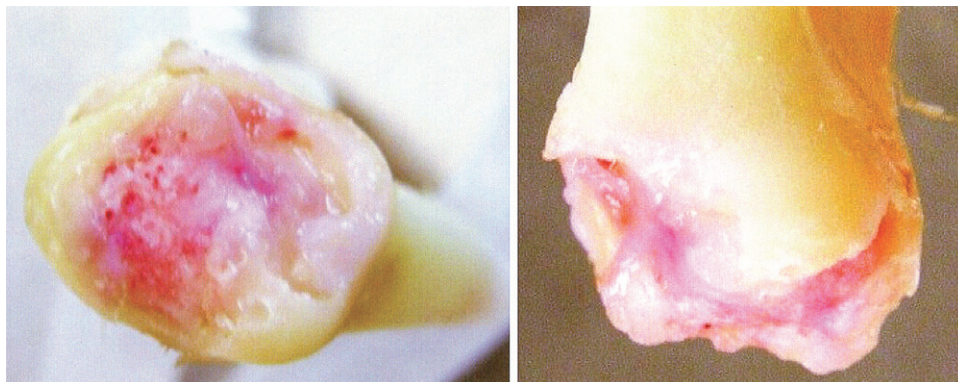


Figure 19.3 Extracted maxillary permanent first molars with crown resorption.

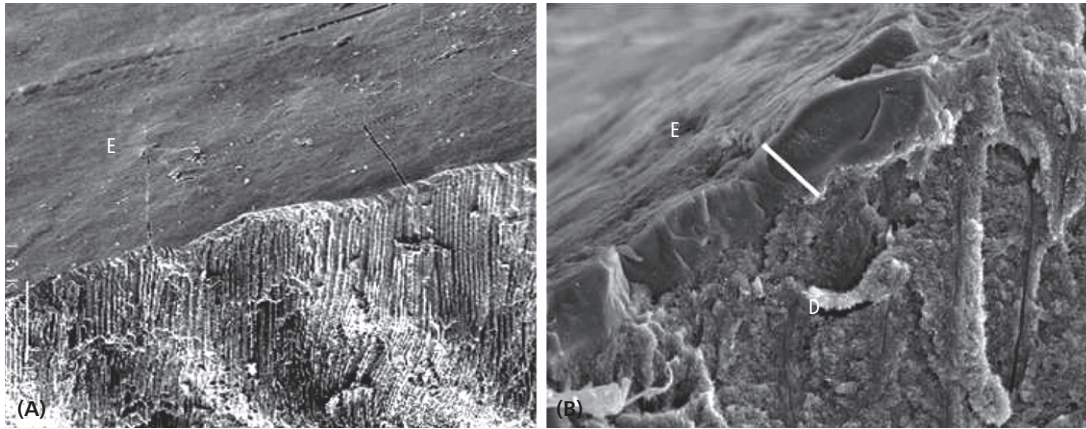


Figure 19.4 Scanning electron micrographs of fractured teeth. A. Normal enamel surface (E) and prismatic fractured enamel layer. B. In the proband's permanent maxillary molar, the enamel lacked any prismatic architecture and was extremely thin. Line $\sim 10\mu\text{m}$; E: enamel; D: dentin; and original magnification $1400\times$.

Histological evaluation

The extracted teeth were preserved for evaluation by scanning electron microscopy (SEM). The teeth were photographed and then fractured, mounted on aluminum stubs with silver paint, and coated with Au–Pd for SEM. The fractured surfaces were evaluated to assess the enamel thickness and structure at various magnifications. The SEM images revealed that the enamel lacked a prismatic architecture typical of human enamel and was extremely thin (Figure 19.4), confirming the clinical diagnosis of a hypoplastic enamel defect.

Mutation analysis

Genomic DNA was isolated from saliva. Based on the family history, genetically defective amelogenin genes on the X and Y chromosomes could be eliminated and suspicion fell on other members of the enamel matrix gene family. The ENAM gene was subjected to DNA nucleotide sequencing as it was the most likely candidate gene due to the generalized thin hypoplastic phenotype that has been associated with several different ENAM mutations. The proband was homozygous for enamelin mutation c.1258_1259insAG; both parents were heterozygous for this mutation. This insertion generates a premature stop codon at codon 448 (Hart *et al.* 2003).

Discussion

The proband in this case report was homozygous for the ENAM mutation and had smooth but very thin enamel that was devoid of any pitting or grooving as has been described in several other cases of ENAM mutations (Hart *et al.* 2003). The finding of unerupted permanent molars that had undergone resorption has been reported

in other cases of hypoplastic AI and is most commonly associated with autosomal recessive rough hypoplastic AI. Failure of eruption in individuals with this type of AI may be a result of an abnormality in the molecular control of the eruption process that is known to be at least partially driven by the odontogenic epithelium. Some AI-associated, non-enamel manifestations could result from modifying genes or environmental effects (Collins *et al.* 1999). It is also possible that mutations in various domains of the ENAM gene could alter the phenotype. An example of such a marked phenotypic diversity can be seen in amelogenin gene mutations associated with AI. Here, mutations altering different functional domains of the amelogenin protein are responsible for markedly different enamel phenotypes (Snead 2003; Snead *et al.* 2006). The proband's mother reported no clinical manifestations of AI. However, it is possible that upon microscopic examination, she could have subclinical pitting or hypoplastic enamel defects that were not previously thought to be associated with AI. Such defects have been previously described in individuals who are heterozygous for the ENAM c.1258_1259insAG mutation. Regrettably, no such exam could be carried out. The proband's father also was heterozygous for this mutation and appeared to have the same generalized thin enamel phenotype as seen in the proband. This finding suggests that the generalized thin hypoplastic AI trait associated with the c.1258_1259insAG ENAM mutation is, indeed, transmitted in an autosomal dominant manner, and that there is a highly variable expressivity in people who are heterozygous for this mutation. The phenotype has been reported to be dose dependent with mild localized enamel pitting segregating as a dominant trait (heterozygous carriers) and smooth

hypoplastic enamel segregating as a recessive trait (Hart *et al.* 2003).

The enamel phenotype is typically more severe when both ENAM alleles are defective (Ozdemir *et al.* 2005). As the genetic mutations associated with the different AI types become known, our ability to diagnose accurately the different AI conditions is improving. The similarity in clinical features makes differentiating some AI types at the clinical level difficult if not impossible. Correlation of the genetic mutations with the clinical phenotypes of each AI subtype will be invaluable for managing patients with AI. This knowledge will also allow us to better predict which AI types are associated with comorbid features such as calculus formation and skeletal open bites as well as select the most optimal treatment approach based on the genotype.

Early diagnosis and treatment, including a comprehensive preventive component, are essential for optimizing the clinical outcome for such patients. Selecting an appropriate treatment approach for each AI patient can be extremely complex. Clinical problems common to all AI patients include poor esthetics, tooth sensitivity, and extensive tooth wear. AI management in childhood and adolescence is aimed at improving these conditions. Restorative materials such as resin-modified glass ionomers, compomers, resin composites, and stainless-steel crowns may be required for young patients as interim restorations, while older patients may eventually require full crowns.

SEM analysis in the case of this proband revealed thin enamel with a lack of prismatic enamel structure, leading one to question whether or not conventional bonding-based restorative therapies were appropriate. Seow and Amaratunge (1998) performed acid etching on the extracted teeth of patients with hypoplastic and hypomineralized AI. They concluded that the lack of typical etching patterns in these variants may be the result of abnormal prism structure, and the standard etching time and/or acid concentration may be inappropriate for the abnormal enamel. Although some studies have concluded that there is no relationship between bond strength to acid-etched enamel and etching conditions, these studies were all performed on sound enamel. Acid-etched bonding therapies are frequently applied in hypoplastic AI cases and generally appear to have a reasonable level of success, suggesting that a thin, nonprismatic enamel layer could be sufficient to adequately retain bonded materials. Selecting appropriate treatment options and materials is ideally predicated based upon understanding the type of enamel defect that is present. For example, pretreatment with NaOCl may be used in hypomineralized AI types to improve bonding as these treatments may help remove protein debris (Saroglu

et al. 2006) but may not be appropriate for use with hypoplastic enamel that is well mineralized and does not have increased protein content.

In the past, treatment of AI has generally been provided without knowing the specific genetic defect responsible for the condition. With an increasing number of AI cases now diagnosable at the molecular level, this may change. It may one day be practical and highly beneficial to determine the specific AI genotype and associated AI phenotype before providing treatment in order to optimize preventive and restorative care. Such individualized personal care is the aim of using genomic information to allow practitioners to tailor therapeutic interventions. By studying the outcomes of various restorative procedures for each genotype–phenotype condition, practicing dentists will be able to use gene-based diagnoses to choose among various treatment options and thereby restore the dentition in a way that achieves the best results (Hu & Yamakoshi 2003).

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Cementum

Brian L. Foster and Martha J. Somerman

There is no such thing in nature as a tooth without cementum.

—Dr. Richard Owen, *Odontography* (ca. 1840)

The first microscopic demonstration of cementum on human teeth is attributed to Jan Evangelista Purkinje, though Anders Retzius was working in parallel to describe tooth anatomy circa 1835. However, those esteemed anatomists were not the discoverers of cementum, as identification by others is recorded as far back as 1691, and eighteenth-century anatomists recognized cementum on animal teeth. The father of microscopy, Anton van Leeuwenhoek, identified this peculiar substance on animal roots, easily distinguishable from enamel and dentin. In the nineteenth and into the twentieth centuries, what is now known simply as cementum was variously called *cement*, *crusta petrosa*, and *ossea substantia*, and its identity was confused with that of the dental follicle, as well as with common tartar buildup. Cementum's identity crisis continues to this day as we still ask, "Are cementoblasts just positional osteoblasts?"

Though tooth cementum has been known to exist for 300 plus years and has been subjected to detailed study in humans and copious numbers of animal species, it remains the enigmatic fourth mineralized tissue of the body. Cementum is one component of the periodontium, the supporting tissues hidden below the gum line. While the crown may be the robust anvil of the tooth, the periodontium is its essential foundation; where periodontia are lacking or degraded, tooth loss swiftly follows. Recognition that periodontal tissues hold the potential for regeneration has spurred interest in understanding the molecular biology of root formation and cementogenesis in order to use this knowledge as the basis for

therapeutic strategies. In that sense, it is useful to review where we are now on the matter of cementum; what do we know, and what questions still need attention?

This chapter is a succinct primer on cementum, and the more inquisitive reader is directed to several detailed reviews and exceptional studies of cementum biology for deeper study (Bosshardt & Schroeder 1996; Diekwisch 2001; Bosshardt 2005; Foster *et al.* 2007).

Types of cementum

Traditionally, cementum is classified into two major varieties based on the basis of whether cells are included and on the primary source of collagen fibers. These are the acellular extrinsic fiber cementum (AEFC) or "acellular" type, and the cellular intrinsic fiber cementum (CIFC) or "cellular" cementum. Though both are present as root covering mineralized tissues, it remains unclear whether these two types are facets of the same tissue or even products of the same cell type. These questions will be addressed in this section and those following, and key characteristics of AEFC and CIFC are compared in Table 20.1 for easy reference. Rodent models are staples of tooth development studies, and, for the most part, major events in odontogenesis are conserved from rodent to human. However, differences in developmental timing and ultrastructure for root development have been documented across species, which serves to introduce a note of caution in extrapolating to the human condition.

Acellular cementum

Acellular cementum is a thin layer present on the cervical and middle portions of roots, characterized by lack of cell inclusions and by extrinsically produced collagen fibers that enter from the periodontal ligament (Figure

Table 20.1 Comparison of key characteristics of the two major types of cementum, acellular cementum (AEFC) and cellular cementum (CIFC). Rows are shaded based on general similarities (white) and differences (gray) between acellular and cellular cementum.

Characteristic	Acellular cementum (AEFC)	Cellular cementum (CIFC)
Localization	Cervical portion of tooth roots	Apical and furcation areas of tooth roots
Collagen fiber origin	Extrinsic	Intrinsic
Function(s)	To cement embedded Sharpey's fibers	To bring and maintain teeth in proper occlusal position
Cells	<i>Cementoblasts</i> : Fibroblastic-cuboidal morphology	<i>Cementoblasts</i> : Cuboidal morphology <i>Cementocytes</i> : Round/flat with dendritic processes
Cellular origin	Ectomesenchymal dental follicle—or <i>transformed dental epithelium</i>	Ectomesenchymal dental follicle—or <i>transformed dental epithelium</i>
Interacting tissues	Embedded Sharpey's fibers continuous with PDL No vascular or nerve supply	Some superficial cementocytes remain in contact with PDL No vascular or nerve supply
Mechanism of matrix mineralization	Extrinsic (fringe fibers) at the PDL–dentin interface are mineralized	Cementoid matrix is produced, then mineralized
Mineralization	45–50% hydroxyapatite	45–50% hydroxyapatite
Mechanical properties	Similar to bone in hardness and elasticity	Similar to bone in hardness and elasticity
Collagens	Major: Type I collagen Minor: Types III, V, VI, and XII	Major: Type I collagen Minor: Types III, V, VI, and XII
Major noncollagenous extracellular matrix proteins	Bone sialoprotein (BSP) Osteopontin (OPN) Osteocalcin (OCN): <i>less than CIFC</i> Dentin matrix protein 1 (DMP1): <i>less than CIFC</i>	Bone sialoprotein (BSP) Osteopontin (OPN) Osteocalcin (OCN) Dentin matrix protein 1 (DMP1) Dentin sialoprotein (DSP)
Proteoglycans	<i>Less than CIFC</i>	Decorin Biglycan Versican Lumican

20.1A and 20.1B). Sharpey's fibers from the periodontal ligament (PDL) insert into the AEFC surface at a high density, emphasizing the importance of this cementum type for tooth attachment and the distribution of forces generated by occlusal loading (Figure 20.1H and 20.1I).

Human acellular cementum in a fully formed tooth measures 15 μm or more, while the diminutive mouse molar features a mere 2–5 μm of AEFC. Normally not subject to turnover, acellular cementum thickness continues to slowly increase at a consistent rate by appositional growth, a property that lends itself to forensic age determination by counting cementum annuli (a kind of analog to the use of growth rings to determine the age of trees).

Cellular cementum

The cellular type of cementum is distinguished by embedded cementocytes in a matrix of intrinsic collagen

fibers (Figure 20.1A and 20.1C). CIFC is present on the apical portion of multirooted teeth, and sometimes in the furcation region, depending on species. In addition, this type is often found as part of the healing process after removal of diseased cementum and is referred to as *reparative* or *regenerative cementum* (Bosshardt & Sculean 2009). Cellular cementum is sometimes called *secondary cementum* because it follows acellular cementogenesis, but it is also described as *adaptive cementum*. The latter term gets at the proposed function of cellular cementum, which is to guide and maintain the tooth into its proper occlusal position during eruption. Evidence for this role has been provided by classic labeling experiments of physiologic eruption and axial tooth movement in rodents, as well as studies of super-eruption showing increased cellular cementum and apical bone apposition when teeth are unopposed (Holliday *et al.*

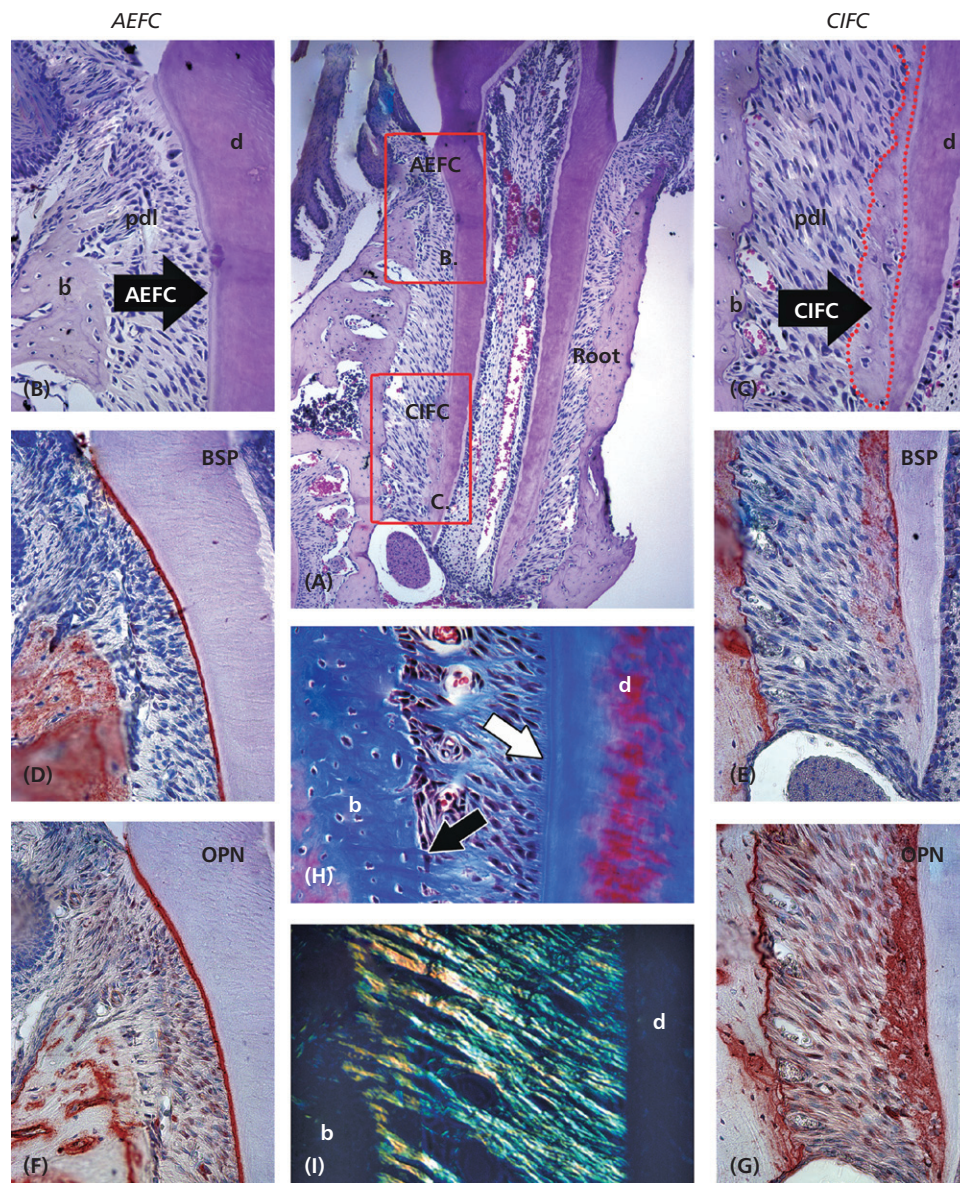


Figure 20.1 Cementum. A. Major varieties of cementum include acellular extrinsic fiber cementum (AEFC; left column) and cellular intrinsic fiber cementum (CIFIC; right column). Inset boxes indicate regions blown up in panels B and C. B. AEFC covers the cervical portion of the root; C. CIFIC (outlined in red) covers the apical portion of root dentin (d). Bone sialoprotein (BSP) and osteopontin (OPN) are concentrated in AEFC, by immunohistochemistry (D and F), while their presence is more diffuse in CIFIC (E and G). H. The importance of AEFC in attachment is emphasized by the high density of Sharpey's fibers inserting into cementum (white arrow) from the periodontal ligament (pdl); they also insert into alveolar bone (b; black arrow). Continuity of ligament fibers from AEFC to bone is emphasized by Masson's trichrome staining. (I) The high density and organization of extrinsic fibers can be visualized by picrosirius red staining under polarized light microscopy. All photos are from mouse first molar cut in frontal (buccal-lingual) sections.

2005). CIFIC likely does not function in attachment because collagen fibers originate primarily from cells within CIFIC, run mostly parallel to the root surface, and do not protrude into the PDL space in large numbers.

Cellular cementum on the apical portions of rodent and human teeth is thick and variable in size. CIFIC has been referred to as bone-like, but it remains avascular and non-innervated, and it is unclear if embedded cementocytes function in tissue homeostasis in a com-

parable fashion to osteocytes. The nature of cellular cementum and its resident cells has not been an area of focused investigation, warranting more attention in light of the new appreciation for the role of osteocytes in bone maintenance.

Other cementums

While AEFC and CIFIC are the major forms of cementum, the cementum family is in fact a little larger; at least

three additional types of cementum have been identified. Acellular afibrillar cementum (AAC) is sometimes found at the most cervical portion of the root, near the cementum–enamel junction (CEJ) or overlapping enamel, and has no known function. Cellular mixed stratified cementum (CMSC) is a composite type composed of alternating layers of AEFC and CIFC and is found along with CIFC on the apical portion of molars. CMSC results from intermittent production of overlapping AEFC and CIFC in response to changes in function during life. Acellular intrinsic fiber cementum (AIFC), featuring neither cells nor protruding fibrils, has been reported on apical portions of human teeth.

To add another complexity, cementum commonly forms on crown enamel of some herbivores (so-called *coronal cementum*) including rabbits, guinea pigs, cows, horses, and elephants. Coronal cementum varies from acellular to cellular depending on species. Animals with continuously erupting teeth (usually incisors, but sometimes molars), including mice, rats, rabbits, and voles, have proven to be useful models for the study of crown versus root development. The mouse, in particular, develops a continually erupting incisor wherein the root analogue (on the lingual aspect) features acellular cementum.

Composition of cementum

Are acellular and cellular cementum distinct tissues, or the same tissue wrought in a different developmental context? Considering the composition of acellular versus cellular cementum provides some basis for comparison of the two tissues to one another, as well as to additional hard tissues of the dentoalveolar complex. Cementum, like bone and dentin, is a mineralized matrix composed largely of collagen, noncollagenous extracellular matrix (ECM) proteins, other organic molecules, and hydroxyapatite (HAP) mineral. Cementum matrix is about 45%–50% mineralized, similar to bone, which is estimated at 50%–60%. More detailed evaluations of cementum composition can be found in previous reviews of the subject (Bosshardt 2005; Foster *et al.* 2007); cementum composition will be considered only briefly here in order to compare key characteristics (Table 20.1).

Collagens

For both acellular and cellular cementum, type I collagen is the major matrix component serving as a scaffold for HAP mineral deposition. Lesser amounts of other collagens, including types III, V, VI, and XII are also present. The remaining organic component of cementum is made up of an assortment of noncollagenous proteins

and proteoglycans, which influence matrix properties and regulate HAP crystal deposition.

Noncollagenous extracellular matrix (ECM) proteins and proteoglycans

The most commonly cited ECM markers for cementum are bone sialoprotein (BSP) and osteopontin (OPN). These acidic phosphoproteins have mineral-regulating properties and are both members of the multifunctional SIBLING (small integrin-binding ligand n-linked glycoprotein) family, which also influence cell migration and attachment. BSP and OPN localize strongly to the acellular cementum and more diffusely to cellular cementum (Figure 20.1D–G). Osteocalcin (OCN), another mineral-regulating protein regarded as an osteoblast marker, is reported in both types of cementum, but more consistently in cellular cementum. Another SIBLING family member, dentin matrix protein 1 (DMP1), is expressed by cementocytes of cellular cementum in parallel to bone osteocytes. DMP1 has, at times, been reported in the cells and matrix of AEFC; however, it is seen more consistently in cementocytes and CIFC (Toyo-sawa *et al.* 2004; Ye *et al.* 2008). The portrait that emerges is one in which these key ECM proteins share overlapping distribution in both types of cementum, bone, and dentin (Bosshardt 2005). In contrast, proteoglycans (PGs), which also influence mineralization, offer more distinction between cementum types. PGs including decorin, biglycan, versican, and lumican have been localized to cellular cementum and bone but not to acellular cementum (Ababneh *et al.* 1999).

Growth and differentiation factors

A multitude of signaling factors are present in the mineralized cementum matrix, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and platelet-derived growth factors (PDGFs). Upon release from the matrix, such factors influence local cells. The presence in cementum of growth and differentiation factors, as well as mineral-regulating enamel matrix proteins (EMPs), becomes interesting in light of efforts to regenerate cementum. One school of thought advises that regenerative approaches should include soft-tissue debridement while aiming to preserve cementum as a means to provide superior regeneration compared to removal of cementum by root planing; this may be attributed to effects of regulatory factors stored in cementum matrix.

Cementum-specific markers

A handful of factors have been described as cementum-specific markers, including cementum attachment protein (CAP), cementum-derived growth factor (CGF

or CDGF), and cementum protein 23 (CP-23), but these show overlapping expression or cross-reactivity with PDL, bone, or other tissues. Sequence analysis suggests CAP and CP-23 are related, collagen-like proteins, while CDGF exhibits mitogenic properties. Even if not exclusive markers, these factors may have roles in cementum physiology and warrant further studies to determine whether they are significant to cementum development.

To summarize, comparison of matrix composition points to both similarities and differences in AEFC versus CIFC, with CIFC exhibiting more parallels with bone. These differences derive from origins of the cells involved, as well as associated regulatory factors, speed of matrix formation, and cell inclusion. Later sections in this chapter are devoted to interpreting the latest developmental evidence to shed light on specific factors regulating cementogenesis.

Cementogenesis revisited: a short primer

Cementogenesis is a late event in odontogenesis, but like earlier stages of tooth formation, it is set in motion by events in the early embryo. Migrating cranial neural crest (CNC) cells stream down from the midbrain and first and second rhombomeres to populate the first branchial arch of the developing embryo. Development of the tooth is driven by sequential, reciprocal, reiterative cross-talk between the odontogenic epithelium and underlying dental mesenchyme, which is ectomesenchyme descended from the migratory CNC cells. Crown development is the result of epithelial-mesenchymal signaling employing major signal families including the BMPs, FGFs, sonic hedgehog (Shh), and Wnt (as detailed in this volume). The product of bud, cap, and bell stages is a crown constructed of enamel and dentin. At this stage, the ectomesenchymal populations of the dental papilla and dental follicle (dental sac), which were continuous during early stages, have now been partitioned by the extended cervical loop of the folded enamel organ. Dental papilla gives rise to the odontoblasts and pulp, and the follicle and loosely arranged perifollicular tissues surrounding the tooth bud presage the periodontia. Through well-designed and carefully analyzed studies taking advantage of microarray technology, relationships between follicle cells and their descendent populations (osteoblasts, PDL cells, and cementoblasts) are becoming more clear (Diekwisch *et al.* 2010). At the culmination of the crown stage, and prior to eruption, root formation is now primed to begin.

Root formation

After completion of crown morphogenesis, the inner and outer enamel epithelia (IEE and OEE, respectively) of the

cervical loop reorganize to form a bilayer structure, Hertwig's epithelial root sheath (HERS). This transition is a key event in odontogenesis in teeth of limited eruption, namely, a switch from crown to root fate in the epithelium, which may be realized through loss or altered regulation of the epithelial stem cell niche and reorganization into HERS (Tummers & Thesleff 2003). Regulation of TGF β signaling plays a large role in whether epithelium goes to a crown (i.e., ameloblast) or root (i.e., HERS) fate (Tummers & Thesleff 2008). Once created and segregated from crown epithelium, HERS cells undergo directed cell proliferation, and the root lengthens. That HERS acts as architect of the nascent root is taken as fact; however, the mechanisms guiding such an undertaking are not understood. A layer of odontoblasts differentiates adjacent to the IEE and produces root dentin contiguous with that of crown (Figure 20.2, panel A).

Acellular cementum formation

During root development, cementum forms in step with the underlying dentin (Figure 20.2, panel B). The origin and nature of cementoblasts remain controversial, as discussed in the “Would the Real Cementoblast Please Step Forward?” section. One of the first events directly contributing to cementogenesis is disruption of HERS to reveal the underlying dentin surface. Mechanisms for fenestration of HERS have been attributed to invasive migration of follicular mesenchyme and self-disruption by cells of HERS. Disruption of OEE and then IEE layers provides access for cementoblast precursors to extend cell processes or invade toward the exposed root dentin surface. Follicle cells have demonstrated substantial migratory capacity during root development in rodents and humans (Diekwisch 2001, 2002). Synthesis of collagen fibrils by these migrating follicle cells contributes to both establishment of Sharpey's fibers and their continuity with dentin matrix fibers to make a strong attachment at the cementum–dentin junction (CDJ) (Bosshardt & Schroeder 1996; Ho *et al.* 2009). Careful study of the structure and materials properties of the CDJ suggests the graded nature of this region contributes to the ability of the root to accommodate occlusal loading, and the CDJ could be considered a fibrous joint akin to the PDL–cementum interface (Ho *et al.* 2007). In human teeth, tightly packed “fringe fibers” are important in continuity of collagen fibers between PDL, cementum, and dentin in the region of acellular cementum (Bosshardt & Schroeder 1996). While cementum is being deposited and PDL fibers reorganizing during root elongation, the alveolar bone surrounding the tooth increases in height and incorporates Sharpey's fibers.

The cementoblasts surrounding the nascent root dentin surface create a local environment conducive to

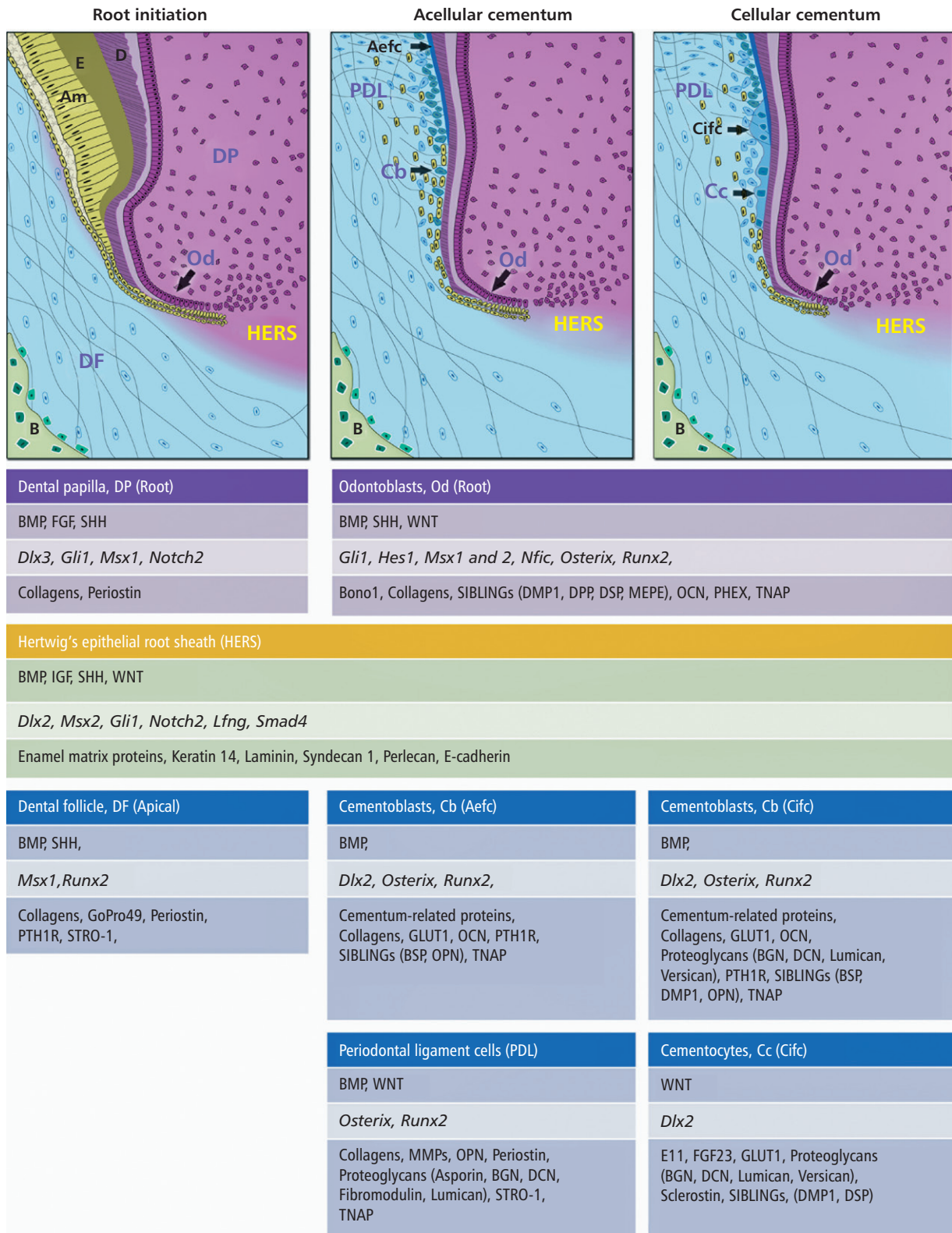


Figure 20.2 Overview of cell signaling involved in tooth root development. For the sake of organization, the continuous process of root development is broken into stages of initiation, acellular cementogenesis, and cellular cementogenesis, which are depicted in the three illustrated panels showing the apical-most portion of the tooth. Key cell populations are labeled and represented by boxes under the illustrations. In these boxes, cells are characterized by major signaling pathways (top row), important transcription factors (middle row, *italics*), and other important characteristic markers (bottom row). The epithelial compartment, which transitions to Hertwig's epithelial root sheath (HERS) at the onset of root development, interacts with adjacent mesenchymal compartments, the dental papilla (DP, which gives rise to odontoblasts (Od) and dentin), and the dental follicle (DF, which gives rise to cementoblasts (Cb) and PDL). While HERS–papilla interactions are well established during root dentinogenesis, hypothesized signaling between HERS or odontoblasts and follicle has not been definitively shown. Factors listed represent data from primary literature, and the list is by no means complete at present. Details and references are provided in the accompanying text. Abbreviations: B: bone; DF: dental follicle; Am: ameloblast; E: enamel; D: dentin; Od: odontoblast; HERS: Hertwig's epithelial root sheath; DP: dental papilla; Cb: cementoblast; Cc: cementocyte; Aefc: acellular extrinsic fiber cementum; and Cifc: cellular intrinsic fiber cementum.

cementum formation (i.e., accretion of HAP mineral on the dentin surface). How is this environment produced? For acellular cementum, extrinsic collagen fibers from the PDL already pass and intermingle with dentin matrix. Cementoblasts produce characteristic noncollagenous ECM proteins including BSP, OCN, and OPN, which are deposited into spaces between collagen fibers and likely influence HAP deposition and crystal growth. The enzyme, tissue-nonspecific alkaline phosphatase (TNAP), which is present in the periodontal region, has been positively correlated to AEFC and is a cementoblast and osteoblast marker. Additional regulatory factors influencing formation of cementum are detailed in this chapter. Cementoblasts responsible for acellular cementum have been described as fibroblastic-cuboidal in morphology and unipolar in their promotion of mineralization, accounting for the slow, controlled nature of acellular cementum synthesis. It has been remarked that acellular cementum seems to form by a process in which mineral is directed and ECM proteins are secreted concomitantly to the root dentin surface. This is unlike dentin, bone, and even cellular cementum, where through a two-step process, a nonmineralized matrix is first elaborated (i.e., predentin, osteoid, and cementoid) and then undergoes gradual mineralization. In this case, acellular cementum could be thought of as a tightly regulated progressive mineralization of the PDL fibers or, more specifically, the fringe fibers of the root surface (Bosshardt & Schroeder 1996; Bosshardt 2005).

Cellular cementum formation

During tooth eruption, the root is partially completed. The process of cementogenesis is altered to produce the thick, more rapidly forming cellular cementum that encases the apical root (Figure 20.2, panel C). Cells producing CIFC are cuboidal in morphology, resembling osteoblasts. A portion of these cells is embedded in the cementoid matrix becoming cementocytes housed in lacunae, which feature a canalicular network of cell pro-

cesses that reach toward the PDL interface. The stimulus for the change from acellular to cellular or mixed types of cementum remains unknown. It has been observed in rodents that CIFC forms approximately at the time the tooth enters occlusion, which is coincident with slowing proliferation of HERS cells. Some have hypothesized that cessation or slowing of HERS growth is a signal for cellular cementum, though how HERS is related to cementogenesis still remains a matter of debate.

What becomes of the HERS cells that guide root formation? Ultimately, the fate of these cells may be apportioned between disaggregation into epithelial cell rests of Malassez (ERMs), apoptosis or other cell deaths, incorporation into cementum matrix, or epithelial-mesenchymal transformation to a mesenchymal cell phenotype (Kaneko *et al.* 1999; Bosshardt 2005; Luan *et al.* 2006). ERMs are small clumps of epithelial cells residing in the PDL, which are interconnected in a fishnet-like network. Waxing interest in ERMs has led to hypotheses for an ongoing role for the cells in some aspects of periodontal maintenance or even cementum regeneration (Rincon *et al.* 2006), though it is currently unclear how this would be accomplished.

Cementum homeostasis

Neither acellular nor cellular cementum participates in remodeling or turnover like that in bone, though cellular cementum undergoes relatively limited resorption and repair events. Some findings have indicated that maintenance of cellular cementum parallels bone in some respects. In a mouse model of hyperocclusion, cellular cementum apposition was disturbed and the tissue was susceptible to resorption, much like the adjacent alveolar bone proper (Walker *et al.* 2008). Orthodontic tooth movement sometimes results in root resorption, which can strike both types of cementum, but more often and more severely destroys apical cellular cementum. As both types of cementum do not undergo measurable turnover and osteoclasts more often target alveolar bone than

cementum in pathological situations, it has been hypothesized that cementoblasts may have a protective, antiresorptive role on the root surface. The role of cementoblasts and cementocytes in osteoclast biology remains to be defined.

Would the real cementoblast please step forward? Controversies on induction and identity of cementum-forming cells

Classical and alternative hypotheses of cementum origins

The origin of the cementoblast remains controversial. The classical hypothesis of cementum origin holds that cementoblasts are derived from the dental follicle that surrounds the developing tooth bud. This hypothesis is in line with the concept that cementum, PDL, and alveolar bone share a common ectomesenchymal origin. According to this proposal, HERS must be disrupted in order to allow cementoblast precursors to pass, contact the root surface, differentiate, and secrete cementum matrix. An alternative hypothesis for cementogenesis posits that epithelial cells from the outer layer of HERS take on a mesenchymal phenotype able to secrete cementum matrix proteins and direct cementum mineralization on the dentin surface. Under the epithelial hypothesis for cementogenesis, acellular and cellular cementum may be derived from different cellular origins, with cellular cementum being generated by an osteoblast-like cell. The potential roles of follicle cells and HERS in cementogenesis are considered below.

A second question of importance regards the proximal signals responsible for directing migration, attachment, and differentiation of cementoblasts. Under the mantle of the classical hypothesis, these roles have been variously ascribed to signals from HERS cells, odontoblasts, or the surface of the mineralized dentin itself. Current knowledge of root- and cementum-related cell signaling is summarized in Figure 20.2, and discussed in the next subsection.

Do HERS cells signal cementoblast differentiation?

The concept that HERS has an inductive role in initiating cementogenesis is appealing in the sense that it parallels well-established epithelial-mesenchymal communication that drives crown formation (and development of other tissues, e.g., limb and hair). It has been speculated that epithelium-derived EMPs such as amelogenin or ameloblastin may play a role in root formation. For decades, numerous investigators have focused on the localization and role of EMPs in HERS or cementum;

however, the literature is rife with both positive and negative reports, as well as various speculations on existing discrepancies. The sporadic localization of EMPs on the root does not make a strong case for an important role for these molecules in root formation (Diekwisch 2001; Bosshardt & Nanci 2004). Mice lacking amelogenin were reported to have resorptive lesions on root surfaces; however, cementum formed normally, and it may be speculated that increased osteoclast activity in the periodontia was secondary to occlusal trauma from poor quality enamel and severe attrition of crowns (Hatakeyama *et al.* 2003). In spite of these lingering questions, a clinical product based on the concept of an epithelial role in cementogenesis was developed and is in clinical use (Bosshardt & Sculean 2009), though it has proven difficult to identify the bioactive component of this mixture that is contributing to tissue repair.

Meanwhile, one instance of epithelial-mesenchymal signaling specifically in root development has been documented in the case of HERS-derived sonic hedgehog (Shh) induction of transcription factor nuclear factor κ B (Nf κ B) in pre-odontoblasts (Huang *et al.* 2009b). Intriguingly, Nf κ B-null mice lack roots altogether (Park *et al.* 2007). HERS induction of root odontoblast differentiation is more in line with epithelial-mesenchymal signaling that occurs in the crown, compared to the idea of a HERS-derived cementogenic factor. Mapping of additional signaling molecules during root formation supports a HERS–odontoblast communication, whereas HERS–cementoblast signaling remains unsubstantiated. For example, dental papilla cells adjacent to HERS express Shh target genes *Patched1* (Ptc1), *Smoothed* (Smo), and *Gli1*, while expression was not localized to dental follicles or cementoblasts (Nakatomi *et al.* 2006). Coincident expression of *Msx2* by HERS and *Msx 1* and *2* and BMPs 2, 4, and 7 by early odontoblasts is suggestive of cross-talk parallel to that in the crown (Yamashiro *et al.* 2003). However, in the same study, cementoblasts and dental follicles did not express *Msx* or BMPs, except for BMP3, an antagonist of BMP signaling. The BMP antagonist *noggin* has been localized to the developing periodontia (Kim *et al.* 2007), and *Noggin* overexpression in epithelia resulted in severe disturbance of root morphogenesis, yet cementum formed (Plikus *et al.* 2005). Other studies report a wider expression of BMPs, transforming growth factor beta 1 (TGF β -1), and Wnt pathway activation in developing periodontia (Tummers & Thesleff 2009; Lohi *et al.* 2010; Rooker *et al.* 2010), and numerous *in vitro* studies demonstrate that such bioactive factors can affect periodontal cell phenotypes. Thus, the major signaling pathways involved in crown formation (BMP, FGF, Shh, and Wnt) are likely involved in

root formation, but specific functions in cementogenesis remain unresolved. In this context, it is also interesting to step back and note that in animals that develop crown cementum (as described in this chapter), exposure of a mineralized surface to cementogenic cells seems to be the precipitating event, as there is no HERS or odontoblast component to provide signals in that milieu.

Do HERS cells become cementoblasts?

What is the evidence that HERS cells may directly secrete cementum matrix proteins and/or transform to a mesenchymal cell? In vitro studies demonstrate potential for HERS-like cells, when properly stimulated, to produce ECM proteins compatible with cementum as well as mineralized nodules (Zeichner-David *et al.* 2003; Sonoyama *et al.* 2007), though this falls short of demonstrating such a physiological role for HERS. Ultrastructural observations have identified HERS cells taking on a secretory morphology, which may be explained by epithelial-mesenchymal transformation (Bosshardt & Nanci 2004).

More direct evidence for cementoblast origins has been provided by lineage tracing experiments used to target specific cells during cementogenesis in mice. Lineage analysis using Wnt1 expression to mark CNC cells and their progeny was built on previous studies over several decades and showed that CNC ectomesenchyme was the primary source for tooth mesenchyme, including the periodontia, though cementogenesis was not studied in detail (Chai *et al.* 2000). A different marker, transcription factor *Dlx-2*, allowed tracking of HERS cells during root formation (Lezot *et al.* 2000). This study demonstrated *Dlx-2* positive HERS cells along the root surface during acellular cementum formation, with a notable sparseness of epithelial cells in cementum-forming regions following disruption of HERS. A role for HERS in cementogenesis was posited, but whether these epithelial cells present on the root surface were active or passively present was not investigated. Another epithelial marker, keratin 14, was used to map HERS cells during mouse root formation (Huang *et al.* 2009a). Keratin 14-positive HERS cells remained closely associated with the developing root surface during the earliest stages of cementogenesis, but increasingly became a minority of cells as AEFC development proceeded. Coupled with results suggesting that some portion of HERS cells lost keratin expression and an overlapping fraction expressed type I collagen, BSP, and TNAP activity, the interpretation was that HERS could transform to a mesenchymal cell type and participate in cementogenesis. That HERS were a minority of root surface cells, and mesenchymal cells were noted to penetrate the

HERS and secrete matrix (consistent with the classical hypothesis), opens the questions of whether multiple cell types contribute to cementum and how such a heterogeneous contribution works to produce such an apparently homogeneous tissue like the acellular cementum.

Contributing to the controversy of cementum origins is the circumstance that there are timing differences in root development of the various animal models used (e.g., mouse and rat vs. human). Diekwisch summed it up eloquently, remarking that “Rodent molar cementogenesis encompasses a particularly dense series of events that might easily generate misconceptions” (Diekwisch 2001). Importantly, in human root development, HERS remains in contact with the newly formed root surface for only a short time, and is then removed to a position several cell layers away as mesenchymal cells invade, all prior to the onset of cementogenesis (Bosshardt & Schroeder 1996; Diekwisch 2001). Insightful comparative anatomy studies of root development in fish, amphibian, reptiles, and mammals consistently associated absence of HERS (by fenestration and removal) with cementum and PDL formation, while species with long-lasting HERS exhibited lack of root cementum in these regions (McIntosh *et al.* 2002; Luan *et al.* 2006). These studies strongly support an evolutionary role for HERS as an inhibitor or regulator of cementogenesis that has evolved to a transitional structure to allow for a gomphosis type of attachment.

Cementogenesis gone awry: insights into the regulation of development and maintenance of cementum

Critical factors for cementum development and maintenance have been identified by pathologies in human patients and, more recently, from transgenic mouse models. Numerous factors have bioactive effects on cementoblasts or precursor cells in vitro; however, the physiologic relevance of such findings remains in question until supported by in vivo observations. Therefore, in vivo studies are the focus of this summary.

Growth factors

Bone and tooth formation, as with other developing organs, is heavily influenced by circulating hormones and growth factors. For review on teeth manifestations, see Foster (2008). In general, cellular cementum is prone to similar defects as disturbed osteoblast differentiation and bone formation. For example, cellular cementum expands in response to overexpression of growth hormone and shows significant reduction when growth hormone or its receptor is lacking, while acellular

cementum is insensitive to such changes (Smid *et al.* 2004). Acellular cementum is relatively less affected than cellular cementum or bone by systemic alterations in hormones and growth factors, though systematic studies of such effects are lacking.

Differentiation factors

To date, woefully little is known about the role or mechanisms of cementoblast differentiation *in vivo*. Dissection of the osteoblast differentiation program continues to uncover important regulators of these related cells. Runx2 was identified as a master transcription factor for the osteoblast program, and inactivating mutations are responsible for the skeletal mineralization abnormalities of cleidocranial dysostosis (CCD). In several case reports going back decades, lack of cellular cementum is associated with CCD, though other reports contradict this. Mice heterozygous for Runx2 feature some disturbance in root development, but histological examination failed to find a developmental cementum phenotype (Zou *et al.* 2003).

Osterix (Osx) operates downstream of Runx2 and is essential for osteoblast differentiation. Osx-null mice lacked bones formed by intramembranous ossification, and initial characterization of conditional Osx KO mice identified lack of cellular cementum, while acellular cementum was unperturbed (J. Feng, personal communication, 2011). Though both Runx2 and Osx have been localized to cells of the acellular and cellular cementum (Hirata *et al.* 2009; Bronckers *et al.* 2001), it remains unclear if they are essential for either tissue. With respect to acellular cementum, it is interesting to speculate on why this tissue might be insensitive to loss of factors like Runx2 and Osterix. An hypothesis has been put forward that cells producing acellular cementum are “partially differentiated,” based on their more fibroblastic morphology and gene expression differences versus cellular cementoblasts and osteoblasts. Lack of effect of ablation of Runx2 and Osterix on acellular cementum certainly provides some support for this view. However, other evidence such as cementum disturbance resulting from Col1-driven BMP4 ablation makes this conclusion less clear (Gluhak-Heinrich *et al.* 2010).

In cellular cementum, the embedded cementocytes are considered the terminally differentiated cell type. The cementocyte of the CIFC is a little understood cell, but breakthroughs in osteocyte biology may provide a stepping stone for understanding these cells and their role in cementogenesis. DMP1 is a multifunctional matrix protein highly expressed by osteocytes, also reported in cementocytes. Ablation of the Dmp1 gene resulted in a defective canalicular system in osteocytes of long and alveolar bones, and similarly defective

cementocytes, explaining a tendency for periodontal degradation (Ye *et al.* 2008). This points to an important role for cementocytes in cellular cementum maintenance.

Mineralization regulators

Classic hormonal regulators of mineral metabolism, vitamin D and parathyroid hormone, can affect all mineralized tissues of the dentoalveolar complex when homeostasis is severely disrupted, though direct roles in cementogenesis are not clear. A rekindled interest in the role of phosphate (P_i) metabolism in physiologic mineralization and pathologic processes has led to study of transgenic mice with dysregulated P_i homeostasis, for example the hypophosphatemic Hyp mouse (mutation in phosphate regulating gene with homology to endopeptidases on the X-chromosome, Phex) and hyperphosphatemic fibroblast growth factor 23 (Fgf23) KO mouse. While both Hyp and Fgf23 KO mice have severe effects on bone and dentin, only relatively mild effects have been identified in acellular cementum, including alterations in ECM protein distribution (Foster *et al.* 2008; Chu *et al.* 2010). Another hypophosphatemic mouse model lacking Dmp1 features an apparently more severe acellular cementum phenotype (Ye *et al.* 2008). Variation in influence of P_i deficiency on acellular cementum indicates that there may be a minimum P_i concentration required for cementogenesis to proceed correctly.

In contrast to P_i metabolism, which contributes to HAP mineral production, pyrophosphate (PP_i) is a potent inhibitor of HAP precipitation and thus dictates physiological and pathological mineralization processes. Cementum was linked to PP_i metabolism through the condition hypophosphatasia (HPP), an inherited deficiency in tissue-nonspecific alkaline phosphatase (TNAP), which hydrolyzes PP_i to P_i . HPP case reports revealed compromised periodontal attachment resulting in exfoliation of teeth. Studies in mice lacking TNAP revealed that AEFC was almost completely abolished (Beertsen *et al.* 1999). Intriguingly, cellular cementum was found to be relatively unaffected, a trend also found in human HPP subjects (van den Bos *et al.* 2005). The sensitivity of cementum to lack of TNAP is well demonstrated by a tooth-specific HPP subtype, odontohypophosphatasia, wherein the dental phenotype is the sole manifestation, even though serum biochemistry is aberrant (see the accompanying clinical correlate in Chapter 21, this volume). The importance of PP_i metabolism to acellular cementum formation has been underscored by additional models wherein PP_i levels are reduced. Loss of function of either progressive ankylosis protein (ANK) or ectonucleotide pyrophosphatase/phosphodiesterase I

(NPP1) causes several-fold expanded acellular cementum, while cellular cementum, dentin, and bone appear unaltered (Nociti *et al.* 2002; Foster *et al.* 2010).

This phenotypic pattern is in line with the account of acellular cementum as a mineral tissue lacking an unmineralized precursor, as opposed to cellular cementum, bone, and dentin. The portrait that emerges of acellular cementum is one of a tissue that depends on the creation of physiochemical environment conducive for apposition, while other mineralized tissues may be more regulated by the acts of cell differentiation and matrix production. This concept is well supported by rodent studies employing PP_i analogs (bisphosphonates) that block *de novo* acellular cementum, while the other hard tissues are produced as unmineralized matrices (Takano *et al.* 2003). A strikingly similar pattern emerged when mineralization inhibitor matrix gla protein (MGP) was ectopically expressed in bones and teeth; bone, dentin, and cellular cementum matrices were produced yet remained unmineralized, while AEFC was completely absent (Kaipatur *et al.* 2008). It must be noted that all evidence suggests cementum formation requires an underlying mineralized dentin to initiate, and so one of the difficulties in identifying factors critical for cementogenesis is that any negative impact on dentin raises the question of whether the cementum effect was primary or secondary.

Based on differences in morphology, rate of formation, ECM protein distribution and composition, and variant phenotypes in response to transgenic manipulation, the different regulatory mechanisms governing acellular versus cellular cementum are becoming clearer.

Medical and health perspectives on periodontal regeneration

Periodontitis leads to destruction of periodontal tissues, including alveolar bone, PDL, and cementum. Predictable regeneration of the periodontium to normal structure and function is a desirable goal that is difficult to achieve. It has been recognized for decades that cells migrating to or present in the local periodontal region have the capacity to foster regeneration of tissues lost as a consequence of disease. More recent evidence corroborates that such cells have stem-like properties (Seo *et al.* 2004), and significant research efforts have been focused on identifying the ideal protocols and agents for use in regenerative therapies. To date, there has been moderate success in periodontal regeneration with the use of a wide range of agents including bone grafts, bone graft substitutes, antimicrobials, biologics, and biomimetics (Wang *et al.* 2005; Reynolds *et al.* 2010). Additional promising agents are currently under investigation or

have the potential for periodontal applications based on success with other bone disorders (Miller 2009; Bashutski *et al.* 2010; Lane & Silverman 2010; Moore *et al.* 2010).

The materials in use and under investigation address the need to provide agents that can promote cellular activities linked to wound healing, repair, or regeneration of the periodontal apparatus, for example mitogenesis, migration, adhesion, spreading, secretion of ECM conducive to mineralization, cell differentiation and maturation, inhibition or modulation of immune responses, epithelial cell down growth, bacterial invasion or growth, and ankylosis of bone and tooth. A biocompatible delivery system for selected bioactive agent(s) will be required in order to release factors with spatial and temporal resolution; this is a practical consideration in order for cells in the area to respond as required for tissue reconstruction. For example, mounting evidence supports the concept that bone and cementum form and regenerate at different rates and thus would have different requirements for optimal regeneration. Even with improved agents and scaffold design, there may be an insufficient number of cells at the site for activation, and thus strategies to provide additional PDL stem-like cells, perhaps from a site proximal to the diseased area, are needed.

Human and animal studies show in principle that regeneration of the periodontium is possible; however, the complex nature of the soft–hard tissue interfaces has proven difficult to restore predictably. Increased understanding of cells and factors participating in root and cementum formation will better inform attempts at periodontal regeneration, as well as the nascent enterprise of tooth bioengineering.

Summary

The cementum that envelops the tooth root is a thin, mineralized tissue providing interface between the tooth proper and the supporting periodontal tissues. Two major types have been identified, acellular and cellular cementum, which carry out distinct functions. Acellular cementum cements principal fibers of the periodontal ligament to the root surface, anchoring the tooth. Cellular cementum provides an adaptive role in guiding the tooth to its occlusal position. While debate continues on the origin of cementoblasts and differences between cementum types, regulatory mechanisms for root formation and cementogenesis have begun to be elucidated through transgenic mouse models and *in vitro* approaches. Studies underscore different developmental regulation of acellular and cellular cementum, thought it remains an open question whether they are distinctive tissues or two facets of the same tissue. Knowledge

gained from these ongoing efforts serves to inform efforts at periodontal regeneration and tooth bioengineering in the years to come.

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Clinical correlate: case study of identical twins with cementum and periodontal defects resulting from odontohypophosphatasia

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Hypophosphatasia (HPP) is an inherited disorder characterized by defective bone and tooth mineralization and deficiency of serum alkaline phosphatase activity (ALP). The disease is a consequence of mutations in the liver, bone, and kidney alkaline phosphatase gene (*Alpl*; OMIM 171760) encoding tissue-nonspecific alkaline phosphatase (TNAP). Biochemical manifestations include increased urinary phosphoethanolamine (PEA), increased plasma pyridoxal 5'-phosphate (PLP), and increased urinary pyrophosphate (PP_i). While PEA, PLP, and PP_i may be physiological substrates for TNAP, accumulation of the mineralization inhibitor PP_i has been identified as the proximal cause for mineralization defects (Whyte 1994).

HPP is highly variable in onset and severity, ranging from stillbirths lacking mineralized bone to milder forms marked by late-onset bone complications or tooth defects without bone complications. HPP has been associated with more than 200 different mutations in *Alpl*, and mode of inheritance can be autosomal dominant or recessive, though recessive patterns are more often identified and are associated with more severe symptoms. Prevalence of severe forms has been estimated at 1/100,000, while the incidence of mild-to-moderate forms is probably higher and more likely to be undiagnosed. HPP is currently classified into six clinical subtypes: perinatal (lethal), infantile, childhood, adult, odonto-HPP, and a rarer, benign prenatal form (Table 21.1; Mornet 2007; Mornet *et al.* 2010). Most HPP sub-

types feature dental abnormalities of varying severity (Reibel *et al.* 2009). Odonto-HPP is distinguished by premature exfoliation of fully rooted primary teeth in the absence of other skeletal manifestations. The anterior deciduous teeth are most likely to be affected, with incisors lost earliest and most frequently. The primary lesion involves a qualitative and quantitative defect of tooth root acellular cementum formation, though root resorption has also been reported (Bruckner *et al.* 1962; Chapple 1993; van den Bos *et al.* 2005). Oral findings may represent the primary clinical manifestations in mild HPP cases in childhood, adult, and odonto-HPP subtypes, and, therefore, premature exfoliation of primary teeth should be a trigger sign that may lead to a clinical diagnosis (Reibel *et al.* 2009).

Case presentation

Two-year-old male identical twins of Caucasian descent were brought for dental evaluation by their parents to the University of Campinas, Dental School at Piracicaba, Brazil. The parents reported premature exfoliation of the anterior primary teeth, with signs of partial root resorption. Examination indicated developmentally normal children with no additional aberrant physical findings. Biochemical analysis revealed low serum ALP activity for both children (patient A: 62 U/L, patient B: 63 U/L; the normal range for children is 151–471 U/L), while serum phosphate and calcium levels were within the normal

Table 21.1 Clinical subtypes of hypophosphatasia.

Clinical subtype	Bone symptoms	Dental symptoms and features	Diagnostics
Perinatal lethal	Severe hypomineralization Osteochondral spurs on forearms and legs Rachitic ribs and respiratory complications	N/A	Ultrasonography Radiographs
Prenatal benign	Bowing, shortening of long bones Improvement of symptoms in third trimester or postpartum	N/A	Ultrasonography Clinical examination
Infantile	Generalized hypomineralization Bowing, shortening of long bones Craniosynostosis Rachitic ribs and respiratory complications Hypercalciuria	Premature loss of deciduous teeth Crown and root developmental defects (see "Odonto-HPP" description)	Clinical examination Radiographs Biochemistry
Childhood	Short stature Development of skeletal deformities Delayed walking, waddling gait Fractures and bone pain	Premature loss of deciduous teeth Crown and root developmental defects (see "Odonto-HPP" description)	Clinical examination Radiographs Biochemistry
Adult	Stress fractures of the metatarsal and tibia Possibility of osteoarthritis or chondrocalcinosis	Possible premature loss of deciduous teeth Possible loss of permanent teeth Crown and root developmental defects (see "Odonto-HPP" description)	Clinical examination Biochemistry
Odonto-HPP	Loss of alveolar bone (may be secondary to tooth defects)	Premature loss of deciduous and/or permanent teeth Delay in eruption Lack of acellular cementum Dentin–pulp defects: reduced dentin thickness, enlarged pulp chambers Enamel hypoplasia, crown abnormalities, dental caries	Clinical examination Radiographs Biochemistry

N/A: Not applicable.

range. Renal function, as evidenced by blood urea, creatinine, and urinalysis, was normal for both patients. Radiographs of the hands, wrists, and legs revealed age-appropriate growth and development, with well-preserved joint spaces. Based on the available clinical, radiographic, and serum biochemistry data, a diagnosis of odonto-HPP was established.

Mutation screening was performed by sequencing the 12 exons of the *Alpl* gene. A heterozygous transition 454C > T in exon 5 resulting in the substitution of arginine by cysteine at position 135 (R135C) was identified in both patients (Weiss *et al.* 1988). This alteration was of maternal origin no paternal mutation was found. While this change may be related to HPP, no correlation with ALP activity, clinical observations, or family historical data has yet been confirmed. This particular altera-

tion has not been previously reported for odonto-HPP, though codon 135 has been linked to a case of adult-onset HPP (R135C/R167W, SESEP Laboratory and the Human Molecular Genetics laboratory of the University of Versailles–Saint Quentin en Yvelines, France), as well as to a lethal HPP case (R135H) (Taillandier *et al.* 2001), suggesting genotype–phenotype correlation. *Alpl* mutations and deletions associated with the odonto-HPP subtype have been localized to exons 4–12, indicating no specific locus associated with this subtype.

The dental history indicated that patient A lost his lower-left primary central incisor (tooth 71 in FDI notation) and patient B lost his upper-left primary central incisor (tooth 61) at approximately age one. Losses were initially attributed to trauma to the mouth caused by falls. About six months following the first incident,

remaining teeth were moderately mobile, and both children experienced additional spontaneous tooth exfoliation at intervals of about six months. Oral examination did not reveal any gingivitis or bleeding on probing. Remaining teeth for patient A included 55, 54, 64, 65, 75, 73, and 85 (A, B, I, J, T, R, and K in the Universal Numbering System). Remaining teeth for patient B included 55, 53, 63, 65, 75, 73, 83, 84, and 85 (A, C, H, J, T, R, M, L, and K). For both patients, multiple teeth featured enamel hypoplasia. Radiographs revealed premature root resorption in the remaining deciduous teeth but apparently normal development of the permanent dentition.

Both patients received partial dentures at three years of age immediately after the loss of their primary teeth to stop the rapid mesial migration of adjacent teeth. From age three years and up, recall visits were scheduled four times per year to monitor general oral health and update the treatment plan for any specific complications. All primary teeth were lost by age seven years (the normal range is age 6–12 years), and eruption of some permanent teeth was delayed. At 14 years of age, patient A experienced loss of his permanent mandibular left central incisor (tooth 31) during brushing (Figure 21.1). The associated radiograph exhibited delayed the erup-

tion of several permanent teeth, which normally erupt between age 10 and 12 years. Scanning electron microscopy on tooth 31 revealed cementum aplasia or severe hypoplasia, with large portions of root dentin exposed. At 19 years of age, patient A suffered an athletic trauma fracturing mandibular right central incisor (tooth 41; Figure 21.2). The lower teeth were splinted with a provisional replacement for tooth 41, and lateral incisors 42 and 32 were treated endodontically because of pulp necrosis. Radiographs demonstrated reduced alveolar bone as well as short roots and wide pulp chambers.

At age 15, patient B suffered athletic trauma resulting in the loss of tooth 31 and pulp necrosis in the maxillary left lateral incisor (tooth 22). At that time, oral examination of both patients revealed relatively good oral hygiene with small amounts of supragingival calculus at tooth–gingival margins. The patients remained on supportive periodontal therapy with recall appointments every three months. Periodontal examinations included assessment of oral hygiene, gingival inflammation, probing depth (PD), clinical attachment loss (CAL), and radiographs taken as appropriate. Treatment included oral hygiene instructions and nonsurgical mechanical debridement.

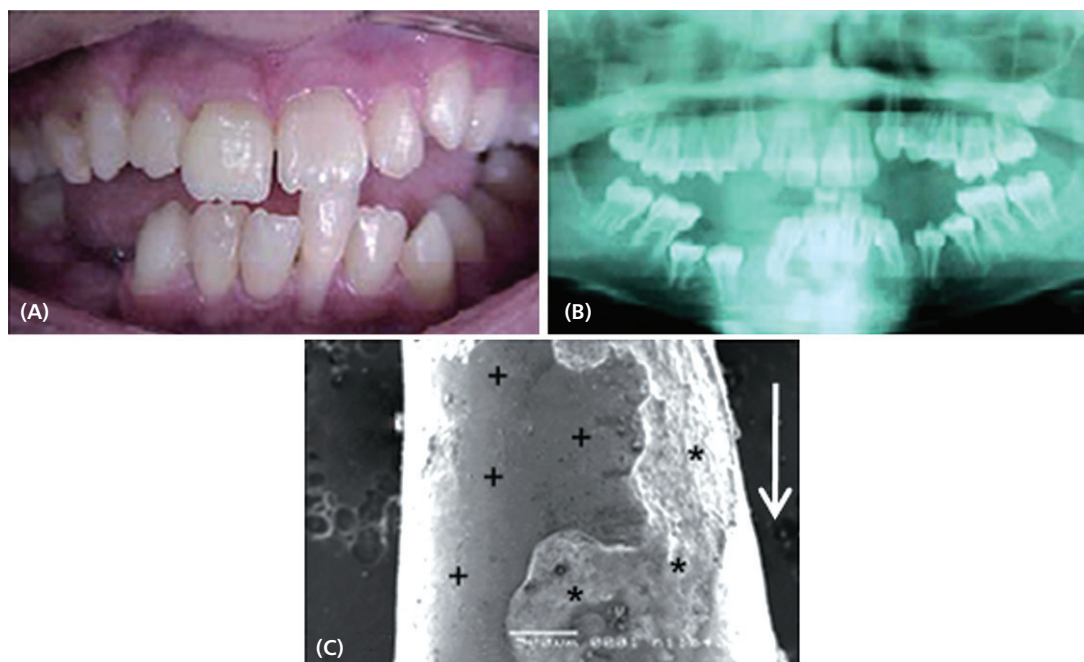


Figure 21.1 At 14 years of age, patient A experienced spontaneous exfoliation of the permanent mandibular central incisor (tooth 31) during brushing. The clinical photograph (A) and corresponding panoramic radiograph (B) were taken prior to tooth exfoliation and show effects of odonto-HPP on the permanent dentition, including delayed eruption of several permanent teeth (e.g., mandibular left and right premolars 34, 35, 44, and 45), enlarged pulp chambers, and thin dentin. The SEM imaging of the apical root region of the central left incisor (tooth 31) shows the lack of cementum and exposed dentin surface with no indication of attached collagenous PDL fibers (+), as well as evidence of root dentin resorption (stars). Arrow indicates the occlusal direction.

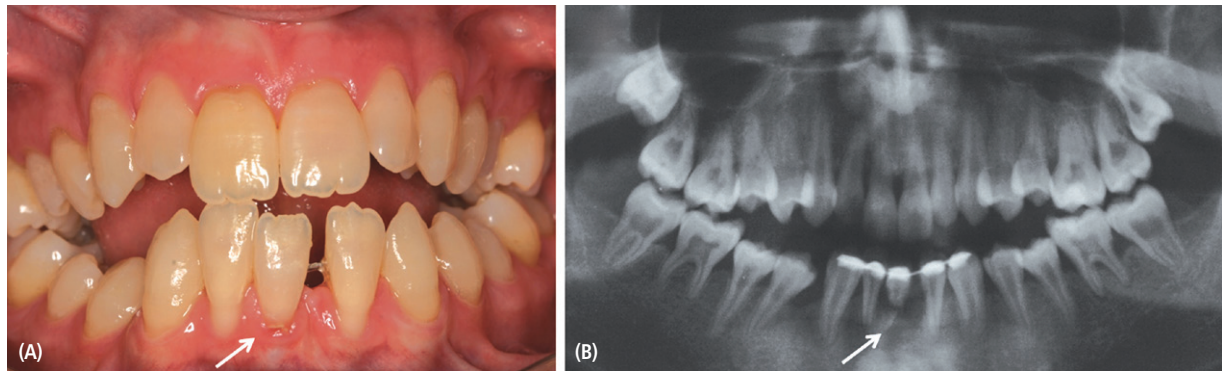


Figure 21.2 At 19 years of age, patient A suffered a fracture of the mandibular right central incisor (tooth 41) as a result of athletic trauma. A. Clinical photograph showing malocclusion and open bite. B. Corresponding panoramic radiograph displaying the fractured tooth (white arrow) and areas of reduced alveolar bone height, as well as short roots and wide pulp chambers.

At age 20, more critical periodontal conditions were found. Bleeding on probing (BOP) was observed in 32% and 13% of the assessed sites (buccomesial, buccocentral, buccodistal, and lingocentral), 8.6% and 3.8% of sites presented CAL of 3–4 mm, and 9.6% and 4.8% sites presented CAL ≥ 5 mm for patients A and B, respectively. Radiographically, reduced alveolar bone height was apparent in the regions of the incisors, while in the posterior region, bone loss was more significant in the area of the first molars, with the mandibular molars extensively involved (Figure 21.3). At this time, compliance was not satisfactory and, therefore, recall appointments were scheduled every two months and included scaling and root planning, oral hygiene instructions, and patient education and motivation.

At present, after two years of this therapy regime, periodontal status remains stable with full-mouth bleeding score and full-mouth plaque score less than 15% for both individuals. With a more stable periodontal condition, for example, minimal bleeding on probing and probing depths ≤ 3 mm, orthodontic treatment was initiated in both patients in order to improve their occlusions after tooth loss and to prepare them for dental implants. The ongoing orthodontic treatment employs minimal forces, with visits scheduled on a monthly basis. Some improvement has already been noted in the patients' occlusions (Figure 21.4). After orthodontic treatment is completed, both patients will be given implant-supported prostheses to replace lost teeth. Biochemical follow-up exams showed that serum ALP activity remains low for both patients at 8U/L and 6U/L, for patients A and B, respectively (normal range for adults is 25–100 U/L).

Discussion

Phosphate (P_i) homeostasis is critical to the normal development, maintenance, repair, and regeneration of

mineralized tissues, including teeth (Foster *et al.* 2008). In the context of mineralization, P_i metabolism is tempered by the actions of pyrophosphate (PP_i), a potent inhibitor of hydroxyapatite crystal precipitation. Studies in recent years indicate that the diverse mineralized tissues of the skeleton and teeth (i.e., bone, cementum, dentin, and enamel) are subject to differential regulation by prevailing P_i/PP_i conditions (van den Bos *et al.* 2005; Foster *et al.* 2008). Cementum of the tooth root is especially sensitive to dysregulation of local PP_i homeostasis based on dramatic phenotypes observed in transgenic mice in which PP_i levels are increased or decreased (Beertsen *et al.* 1999; Nociti *et al.* 2002; Foster *et al.* 2008).

Reduced TNAP function in HPP results in defective bone and tooth mineralization, with severity of clinical presentation associated with dominant or recessive inheritance, age of onset, and the extent of reduction of ALP activity (Whyte 1994; Mornet 2007). HPP provided the first clue linking PP_i metabolism disorder with a developmental cementum phenotype. TNAP deficiency causes aplasia or severe hypoplasia of the acellular cementum (acellular extrinsic fiber cementum (AEFC); Bruckner *et al.* 1962; Chapple 1993; van den Bos *et al.* 2005). Acellular cementum is critical for tooth attachment as it anchors the root to the surrounding alveolar bone via the periodontal ligament (PDL). When AEFC is deficient, as in HPP, Sharpey's fibers from the PDL are poorly developed and insecurely embedded, which results in increased tooth mobility and susceptibility to exfoliation. The rich expression of TNAP in periodontal tissues, as well as developmental analysis of Akp2 (mouse homologue of Alpl) knockout mice, has further highlighted the importance of local expression of this enzyme for initiation of cementogenesis (Groeneveld *et al.* 1995; Beertsen *et al.* 1999). HPP affects other tissues of the dentoalveolar complex (Table 21.1). Alveolar bone

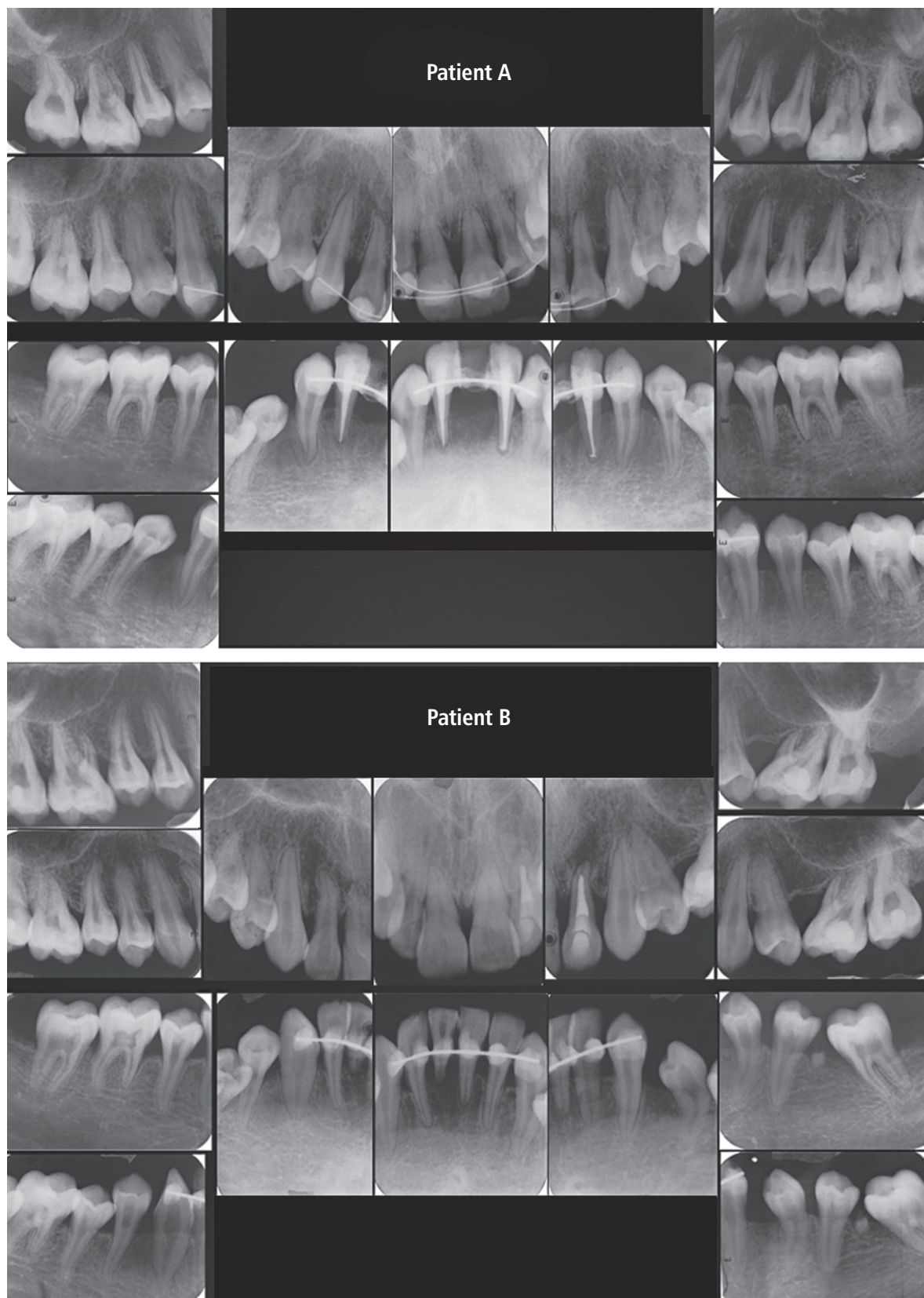


Figure 21.3 Full-mouth periapical radiographs of patients A and B taken at 20 years of age showing reduced alveolar bone height, short root length, and abnormally wide pulp chambers and root canal systems in several teeth. Note that teeth splinting was necessary for both patients and that endodontic treatment was performed because of pulp necrosis. For patient B, a root tip was evident in the molar region of the lower left side, which was later extracted. Although there was an evident and significant amount of bone loss observed for both individuals with furcation involvement in some teeth, probing depth was always ≤ 3 mm with minimal bleeding.

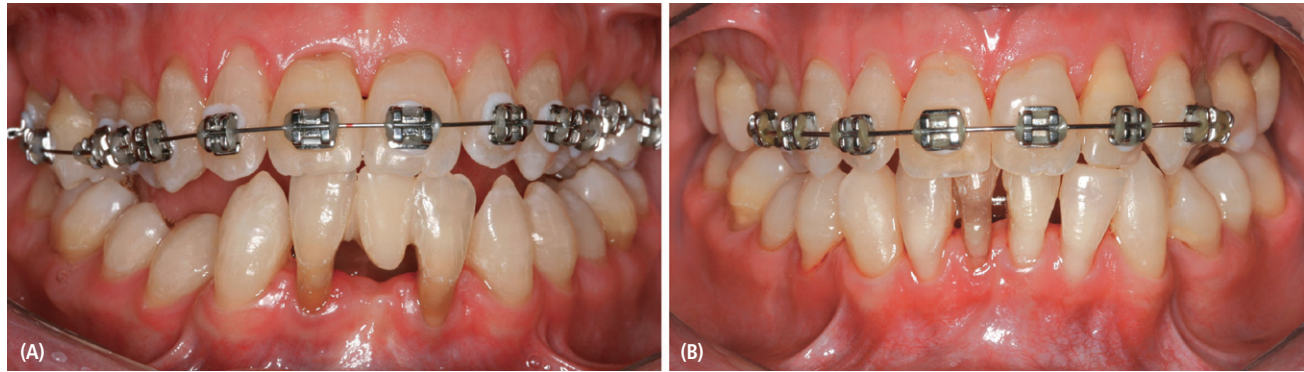


Figure 21.4 Clinical photographs of patient A and B taken after periodontal treatment and during the active phase of orthodontic treatment.

mineralization may be disrupted by loss of ALP activity resulting in hyperosteoidosis, and be secondarily affected by exfoliation of teeth and loss of occlusal loading. Some case reports have described dentin defects including short roots, thin dentin, and abnormally wide pulp chambers, as well as enamel hypoplasia and susceptibility to caries (Hu *et al.* 2000; Reibel *et al.* 2009). Currently, no effective treatment exists for HPP, but TNAP enzyme replacement therapy looks promising for both skeletal and dental defects (Millán *et al.* 2008).

In the two cases reported here, premature loss of the primary dentition served as a trigger sign for diagnosis of HPP. Decreased serum ALP activity and lack of additional mineralization disorders narrowed the focus to the odonto-HPP subtype. The patients presented additional features suggestive of odonto-HPP, including reduced alveolar bone height, enlargement of coronal and root pulp chambers, enamel hypoplasia in primary teeth, and delayed eruption of permanent teeth. SEM imaging of one of the exfoliated permanent teeth confirmed cement defects and extensive root resorption.

Odontohypophosphatasia presents a complex challenge for clinicians. The pediatric dentist faced with early signs of odonto-HPP is uniquely positioned to guide early diagnosis and initiate conservative care of affected patients. However, the difficult questions are: what prognosis is expected and, correspondingly, and what manner of treatment is recommended? The variable severity of HPP makes these difficult questions to answer, though case reports highlighting long-term dental care of HPP patients may provide some guidance on these issues. A fair long-term dental prognosis for a patient with infantile HPP and a severe deciduous dental phenotype was reported by Reibel and colleagues (2009). Though this patient lost all deciduous teeth by eight years of age, and the secondary teeth featured crown and root abnormalities, most of the permanent teeth were still present after

20 years of follow-up, and alveolar bone height remained stable. Pulp chambers featured secondary dentin apposition suggesting delayed dentin formation or mineralization and the possibility for partial correction of developmental defects with time. In some cases, permanent teeth seem wholly unaffected, even when a severe phenotype is present in the deciduous teeth (Lepe *et al.* 1997). Conversely, other clinical descriptions of HPP paint a less optimistic picture when both primary and secondary dentitions are severely affected, resulting in a much less favorable outcome (Macfarlane & Swart 1989; el-Labban *et al.* 1991; Olsson *et al.* 1996).

Conclusion

Premature loss of deciduous teeth in the absence of skeletal disorders may serve as a critical trigger sign for diagnosis of odonto-HPP or other subtypes. Thus, the pediatric dentist is uniquely positioned to guide early diagnosis and initiate the kind of systematic and conservative care of affected patients that can successfully maintain permanent teeth over a long period of time. If a degree of periodontal health and stability can be attained, orthodontic treatment and implant-supported prostheses may also be considered to restore function and esthetics lost due to early exfoliation of permanent teeth.

Summary

HPP is a rare inherited disorder caused by mutations in the gene for the tissue nonspecific form of alkaline phosphatase (ALP). HPP is variable in onset and severity and is characterized by a continuum of mineralization defects in bones and teeth, including defective cementum development and early loss of primary and permanent teeth.

Two-year-old identical twin boys presented with premature exfoliation of anterior teeth. Both exhibited low

levels of serum alkaline phosphatase activity but no additional skeletal abnormalities, prompting a diagnosis of odonto-HPP. The twins underwent pediatric dental care and supportive periodontal therapy for the next 19 years, which was aimed at avoiding or delaying loss of their permanent teeth. Both patients are currently undergoing orthodontic treatment in preparation for receiving implant-supported prostheses.

Acknowledgments

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Dental engineering: tooth regeneration

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The main component of tooth is dentin, a hard and avascular tissue that can withstand the forces of mastication. Dental pulp, the soft tissue fully enclosed in dentin, provides nutrition to the tooth, acts as biosensor of the environment, and offers limited reparative potential to damaged dental tissue. Enamel is the thin layer of very hard tissue covering the dentin at the tooth crown. The tooth root dentin is covered by the periodontium, which contains multiple tissues including cellular and acellular cementum, periodontal ligament (PDL), and alveolar bone lining the tooth socket. The PDL not only anchors the tooth to the surrounding alveolar bone, but also serves as a cushion to buffer the forces of mastication. The PDL contains stem cells that contribute to the repair and remodeling of the periodontium.

Importance of tooth regeneration

The main function of teeth is mastication, thereby providing nutrition. Teeth also facilitate speech and contribute to facial aesthetics. Tooth loss can severely affect quality of life, including an individual's self-esteem, and is still considered a major health issue. Conventional therapies for treating tooth loss are dentures and implants. Currently, the 10-year prognosis for dental implant therapy is quite stable (Shalabi *et al.* 2007; Atieh *et al.* 2010). Therefore, dental implants are widely used to treat tooth loss. Bone and cementum formation is commonly observed at the interface between bone and dental implant, which is termed *osseointegration* (Brånemark *et al.* 1977). This ankylosis-like connection provides a close and tight contact, such that the forces of mastication are transferred directly from the implant to the surrounding alveolar bone (Rinaldi & Arana-Chavez

2010), unlike natural teeth in which the force is cushioned by PDL tissues. Thus, rigid osseointegration can cause damage and absorption of the surrounding bone, resulting in implant failure. Moreover, without the PDL, it is difficult to adjust the position of the implant, if necessary (Devlin & Sloan 2002).

Since the dental pulp is fully enclosed in hard dentin, it is difficult to access the pulp cavity and to insure the complete removal of inflammatory pathogens caused by infection. For this reason, endodontic therapy, which involves the complete removal of the pulp and replacement with synthetic materials, is a common therapy even when inflammation is present in only a portion of the pulp. The success rate of endodontic treatment is high, and the teeth can survive a long time after treatment. However, synthetic pulp material is not vascularized or innervated, and therefore lacks the ability to sense stimuli from the environment, making the tooth susceptible to further damage. In addition, endodontic treatment can cause the tooth to become brittle over time, often eventually requiring tooth extraction. Lastly, synthetic materials have no capacity for self-repair. For these reasons, bioengineered dental tissues would provide a better means for vital, long-term tooth repair or replacement.

Tooth development

Tooth development begins as thickening and invagination of the oral epithelium, and subsequent condensation of the underlying dental mesenchyme. Subsequent continued interactions between the dental epithelial and mesenchymal cell layers direct the formation of functional teeth. Eventually, dental epithelial cells differentiate into enamel forming ameloblasts, while dental

mesenchymal cells differentiate into cells that form the remaining tissues of the tooth and supporting bone. The tooth morphology is controlled by the enamel knot signaling centers, which direct the location and number of tooth cusps; then, signals that direct tooth morphology are transferred from oral epithelium to the dental mesenchyme. Tooth root formation is initiated by the elongation of the Hertwig's epithelial root sheath (HERS), and eventually form mature functional tooth roots and periodontium. A thorough understanding of the signaling events directing natural tooth development greatly facilitates tooth regeneration efforts. The molecular signaling pathways regulating tooth development have been particularly well studied using transgenic mouse knockout models, in which numerous growth factors regulating tooth development, and dental epithelial-mesenchymal cell interactions in particular, have been thoroughly examined. Among these, bone morphogenic proteins (BMPs), sonic hedgehog (Shh), and the wingless-related MMTV integration site gene product (Wnt) family members have been identified as key components for the regulation of tooth development (Thesleff 2006). In contrast, comparatively little is known about tooth eruption, other than the fact that it is related to root formation. This is largely due to the fact that many growth factors are reiteratively expressed throughout tooth development, and mouse knockout models typically exhibit arrested tooth development long before tooth root development occurs.

Tooth regeneration

It is generally accepted that many human tissues have the potential to regenerate due to the presence of adult stem cells in differentiated tissues. A clear definition of tissue engineering was given by Langer and Vacanti (1993), who wrote that tissue engineering is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ." In fact, dental tissue regeneration has an even longer history—the first attempt of periodontal tissue regeneration by guided tissue regeneration (GTR) occurred in 1980 (Nyman *et al.* 1982). Rapid progress in stem cell research over the past decade has established stem cell-based tissue engineering as a promising approach for a variety of clinically relevant therapies. Tooth regeneration is a particularly interesting field because it can solve the problem of tooth repair and regeneration, as well as offer a convenient model for the regeneration of organs of ectodermal origin.

Theoretically, there are three methods for tooth regeneration using tissue-engineering techniques: (1) tooth

bud regeneration, (2) in situ tissue regeneration, and (3) mature dental tissue regeneration. A tooth bud regeneration approach is to bioengineer an early-forming tooth bud, by reproducing the proper spatial and reciprocal epithelial-mesenchymal cell layer interactions. The bioengineered tooth bud can then be implanted into the jaw, at the location of prior tooth loss and undergo further development into a mature and functional tooth. This method is aimed at whole-tooth regeneration (Figure 22.1A). In situ tissue regeneration, also called *cell homing*, occurs when an engineered scaffold is implanted directly into the repair site in order to attract and simulate cells for local tissue regeneration (Figure 22.1B). The third method requires bioengineering mature, living dental tissues in vitro that can be transplanted to the appropriate location. The last two methods are aimed at partial dental tissue engineering and may provide better clinical potential because of the anticipated long-term shelf life.

An important consideration for tooth tissue-engineering strategies is the need to ensure that the interactions that occur between dental progenitor cells, scaffold materials, and growth factors mimic those that occur in natural tooth development. This is essential for successful dental tissue formation, especially for full-sized tooth regeneration. Ideal cell sources for tissue regeneration should not only have the potential to differentiate into specific cell types, but should also be easily accessible and have the capacity for viable and reliable expansion in culture.

Stem cells for tooth regeneration

It is generally agreed that selecting an appropriate cell source is the most important consideration for successful tissue regeneration. Potential cell sources include postnatal stem cells (PSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPS cells).

Postnatal stem cells, also known as *adult stem cells* (ASCs), are resident in a variety of differentiated tissues and organs in both children and adults. In this chapter, the term *PSCs* will be used to refer to all of these stem cell types to eliminate confusion. PSCs have the potential to migrate into the area of damaged tissues, proliferate, and differentiate into all of the specialized cell types of the organ from which they originated. For these reasons, stem cell-based therapies using PSCs are widely considered the ultimate treatment for a variety of diseases, including leukemia, Parkinson's disease, and diabetes (Salem & Thiemermann 2010; Schwarz & Schwarz 2010). Another special property of PSCs is that they can be isolated from the individual themselves, a term that is referred to as *autogenous*. Tissues produced by autoge-

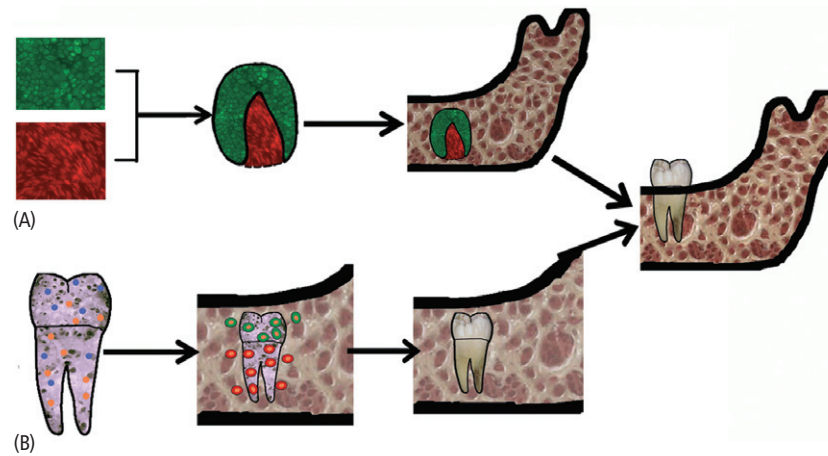


Figure 22.1 Methods for tooth regeneration. A. Tooth bud method. A tooth bud is engineered from dissociated epithelial and mesenchymal cells and transplanted to an in vivo location such as the jawbone. The engineered tooth bud will develop further under the guidance of environment. B. Cell-homing method. A fabricated scaffold of specified size and shape is transplanted into the edentulous area of the jawbone. Stem cells from the environment can be recruited by the pre-integrated scaffold containing growth factors to direct tooth development. Orthodontic treatment or secondary transplantation then is used to move the engineered tooth to a desired location. Green indicates epithelial tissue; red indicates mesenchymal tissue.

nous cells will not cause an immune-rejection response. PSCs origins from specific dental tissues that have been used for dental tissue regeneration include stem cells derived from dental pulp, apical papillae, periodontal ligaments (PDL), dental epithelials, and tooth buds. Nondental tissues stem cells consist of bone marrow-derived mesenchymal stem cells, bone marrow stromal cells (BMSCs), and iPS cells.

Postnatal dental pulp stem cells (DPSCs) and stem cells from human exfoliated deciduous teeth (SHEDs)

Odontoblasts are terminally differentiated cells present in dental pulp that exhibit the ability to synthesize dentin. In response to a strong stimulus such as caries, trauma, and erosion, odontoblasts can be easily damaged and lose their ability to synthesize dentin. However, the formation of reparative dentin under such circumstances, demonstrates the presence of DPSCs in dental pulp, which can be induced to differentiate into odontoblasts (Heyeraas *et al.* 2001). Based on this observation, in the year 2000, it was demonstrated for the first time that stem cells isolated from postnatal human dental pulp could form a dentin–pulp-like complex with a well-defined layer of odontoblast-like cells, dentin, and a highly vascularized pulp tissue center after being transplanted into immunocompromised mice (Gronthos *et al.* 2000). DPSCs were also shown to be capable of neuronal cell differentiation when cultured in the neurogenic medium in vitro (Zhang *et al.* 2006), and dental pulp tissue grafted into hemisected spinal cord increased the number of motoneurons, suggesting that dental

pulp-derived neurotrophic factors may play an important role in orchestrating dental pulp innervations (Nosrat *et al.* 2001). It also was revealed that human pulp fibroblasts harvested from third molar teeth expressed two important proangiogenic factors: vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2; Tran-Hung *et al.* 2006).

Stem cells with properties similar to adult DPSC isolated from human deciduous teeth were named “stem cells from human exfoliated deciduous teeth” (Miura *et al.* 2003). Similar to their adult tooth counterpart, SHEDs also exhibit multilineage differentiation potential, including neurogenic potential, and are able to form a dentin–pulp complex in vivo. SHEDs seeded onto a synthetic D,L,L-poly(lactic acid) (PLGA) scaffold were capable of forming living pulp in endodontically treated teeth (Gotlieb *et al.* 2008). SHEDs were also demonstrated to differentiate into odontoblast-like and endothelial-like cells when seeded onto tooth slices containing a poly-L-lactic acid (PLLA) polymer scaffold-packed pulp cavity (Cordeiro *et al.* 2008).

Together, these results support the idea that DPSCs possess stem cell-like qualities, including self-renewal capability and multilineage differentiation ability. Therefore, DPSCs may be a useful resource for the regeneration of tooth and supporting tissues.

Stem cells from the apical papilla (SCAPs)

In certain cases, immature teeth that have undergone endodontic treatment, have been shown to continue tooth root formation, indicating the existence of a stem cell population at the tooth root apex (Selden 2002).

Stem cells isolated from this region were named *stem cells from the apical papilla*. Similar to DPSCs and SHEDs, SCAPs can also differentiate into odontoblast-like cells and produce dentin-pulp complex in vivo (Sonoyama *et al.* 2006; Abe *et al.* 2008). When compared to DPSCs and SHEDs, SCAPs exhibit even higher in vitro proliferation rates. Furthermore, their location at the apex of the tooth allows them to receive a blood supply from surrounding tissues, which provides them the means to survive after pulp necrosis or endodontic treatment (Huang *et al.* 2008).

PDL

The periodontal ligament has potential for self-healing and regeneration after the removal of local pathogens. Under orthodontic treatment, PDL tissues are constantly remodeling in response to the mechanical stimulus of mastication. Stem cells obtained from PDL tissues (PDLSCs), when seeded onto three-dimensional (3D) scaffolds, exhibit the characteristic ability to form cementum-PDL-like structures in nude mice (Trubiani *et al.* 2008). No published reports have confirmed the ability of PDLSCs to generate a dentin-pulp-like complex.

Dental epithelial stem cells

Some mammals have incisors, and some have molars, that can grow continuously throughout their lives. Analyses of these teeth identified a dental epithelial stem cell niche located at the cervical loop. These resident stem cells exhibited the ability to differentiate into enamel-forming ameloblasts (Kawano *et al.* 2004). Unfortunately for most species, including humans, mature enamel regeneration after tooth eruption is impossible due to the fact that enamel-producing dental epithelial cells have, for the most part, undergone apoptosis prior to tooth eruption. Methods to induce nondental epithelial cells, such as oral epithelial cells, to differentiate into enamel-producing ameloblast cells, could help to overcome this barrier to enamel regeneration therapies.

Dental tooth bud cells

Immature tooth buds contain all cell types required to regenerate all dental tissues. Several groups have reported the formation of bioengineered teeth, with anatomically correct tooth-crown shape containing enamel, dentin, and pulp tissues from re-aggregated tooth bud cells (Hu *et al.* 2006a). Recognizable dental tissues, including dentin, pulp, and enamel, were also observed in dental implants generated from dissociated tooth bud cells seeded onto synthetic polyester scaffolds (Young *et al.* 2002).

Naturally formed teeth have two types of tooth buds based on their developmental stages—embryonic and

postnatal tooth buds. Comparatively speaking, to date, more robust and organized dental tissues have been generated from embryonic tooth bud cells, which are also easier to manipulate in order to manufacture specific tooth morphology (Honda *et al.* 2008). While the study of embryonic tooth bud cell characterizations provides valuable information about the mechanism of tooth regeneration, the general lack of available and suitable autologous human embryonic tooth cells makes these studies of limited use for widely applicable tooth regeneration strategies in humans.

The dental follicle is the tooth sac derived from ectomesenchymal tissue surrounding the developing tooth that participates in the formation of periodontal progenitor cells, including osteoblasts and cementoblasts. The presence of stem cells in dental follicles (DFSCs) has been considered, since they have been shown to be able to differentiate into osteoblasts and cementoblasts, adipocytes, and neurons (Yao *et al.* 2008). DFSCs have been isolated from human third molars, that is, wisdom teeth, and shown to express the putative stem cell markers Notch-1 and Nestin and to produce compact calcified nodules in vitro (Morsczeck *et al.* 2005).

Bone marrow stromal cells

BMSCs are the nonhematopoietic mesenchymal stem cells isolated from bone marrow that can differentiate into a wide range of specific cell types (Chamberlain *et al.* 2007). In the dental field, BMSCs have been commonly used for alveolar bone regeneration. A recent report even demonstrated the differentiation of bone marrow-derived c-Kit⁺ enriched cells into ameloblast-like cells (Hu *et al.* 2006b).

Embryonic stem cells (ESCs)

ESCs are the most versatile of all stem cell types, because they have the ability to differentiate into cells comprising tissues of all three germ layers. However, considerable ethical debate and the possibility for immune rejection, malignant neoplasm, or teratoma formation severely limit their clinic usage. For these reasons, few reports document the use of ESCs in tooth regeneration. It has been shown that human ESCs can facilitate periodontal tissue regeneration in vitro when they are co-cultured with periodontal ligament fibroblastic cells (Inanc *et al.* 2009). Another report demonstrated that mouse ESCs were able to differentiate into ameloblasts when cultured in conditioned media (Ning *et al.* 2010).

Induced pluripotent stem (iPS) cells

Recently, a new type of stem cell, iPS cells, has begun to generate considerable attention for applications in stem cell-based therapies. These iPS cells were generated after

nuclear transfer from fibroblasts to oocytes by the expression of the four factors c-Myc, Klf4, Oct4, and Sox2 (Takahashi & Yamanaka 2006; Takahashi *et al.* 2007) or Lin28, Nanog, Oct4, and Sox2 (Yu *et al.* 2007). Comparison to ESCs demonstrated that iPS cells share similar pluripotent differentiation potential. More importantly, iPS cells can be generated from the patient's own cells, thereby eliminating potential immunologic incompatibility reactions (Yu *et al.* 2007; Park *et al.* 2008). Applications for iPS cells in the dental field have only recently been reported. Duan and colleagues (2011) reported that human iPS cells generated from human foreskin fibroblasts by expressing Oct4, Sox2, Nanog, and Lin28 were shown to promote the formation of new cementum, alveolar bone, and normal periodontal ligament. Two additional publications demonstrated the ability to induce iPS cells from dental cells. Ohnishi's group successfully generates iPS cells by retroviral transduction of OCT3/4, SOX2, and KLF4 to mesenchymal cells from human third molars (Oda *et al.* 2010). Huang's group tested Lin28/Nanog/Oct4/Sox2 or c-Myc/Klf4/Oct4/Sox2 induction on three different dental stem cells: SHEDs, SCAPs, and DPSCs. They reported that all three cell lines not only could be reprogrammed into iPS cells but also showed a higher transfection rate than fibroblasts (Yan *et al.* 2010).

Scaffolds and growth factors are the other two key components in tissue regeneration. Optimized scaffolds for tissue-engineering applications offer both physical and biological support for cells, ideal degradation rates, and eventual replacement by regenerated tissues. To insure suitability for various tissue-engineering applications, scaffolds can be fabricated into different size, shapes, and forms and even more complicated structures with combined materials or intricate inner pores. Numerous natural and synthetic materials have been tested for dental tissue-engineering purposes. So far, the ideal material for dental tissue regeneration has not been identified. Growth factors, when delivered to the wound site and surrounding healthy tissues, benefit tissue regeneration. However, naturally produced growth factors and molecular signals may not be strong enough to elicit robust tissue regeneration. Bioengineering scaffolds that deliver appropriate growth factors or selectively interact with specific growth factor receptors of target cells provide a possible means to facilitate tissue regeneration.

Whole-tooth regeneration

At the present time, both dental- and non-dental-derived stem cells have been shown to exhibit the ability to differentiate into a variety of dental tissues *in vitro* and *in vivo*. The challenge that remains is to devise methods to

achieve the ultimate goal of dental tissue engineering—the ability to regenerate full-sized, fully functional, bioengineered teeth. One essential step required for successful whole-tooth tissue engineering is to devise reliable approaches that recapitulate the proper spatial and reciprocal interactions that occur between epithelial and mesenchymal dental cells during natural tooth development.

Methods using dissociated cells with tooth-shaped scaffolds

The basic concept of this method is to seed dissociated dental cells onto tooth-shaped, biodegradable scaffolds. The dissociated cells can attach to the scaffold and eventually adopt the shape of the scaffold by replacing the scaffold materials as they degrade. Enzymatically dissociated pig tooth bud cells were seeded onto tooth-shaped, three-dimensional polyglycolate/poly-L-lactate (PGA/PLLA) and poly-L-lactate-co-glycolate (PLGA) mold and transplanted into the omentum of athymic rats. After 30 weeks, small but morphologically correct tooth crowns, including dentin, odontoblasts, pulp, and enamel, were detected (Young *et al.* 2002). Similar results were obtained using the same approach but different scaffold materials (Honda *et al.* 2007); multiple small teeth with aberrant cusp morphology formed in one construct, suggesting that the interactions between epithelium and mesenchyme occurred randomly in this model.

Methods using recombined epithelial and mesenchymal layers

This method first generates separate dental epithelial and mesenchymal cell layers and then sandwiches them together to form an artificial tooth bud. Tooth formation was engineered by combining dissociated mesenchymal tooth bud cells with intact dental epithelium and kidney capsule transplantation (Yamamoto *et al.* 2003). Similar results were obtained by combining a dissociated dental epithelial cell layer with a mixture of embryonic neural stem cells from embryo spinal cords and embryonic bone marrow cells (Ohazama *et al.* 2004). Generally speaking, the most successful results were obtained using cells from mouse embryonic tooth buds harvested E11–14.5. A study demonstrated that six-month-old porcine dental epithelial tooth bud cells co-cultured with human dental pulp cells formed soft tissues and expressed certain dental cell markers, although no distinct tooth-like structures were observed (Zhang *et al.* 2006).

Bioengineered organ germ method

A bioengineered organ germ method (Figure 22.2) was used to regenerate tooth buds by seeding dental

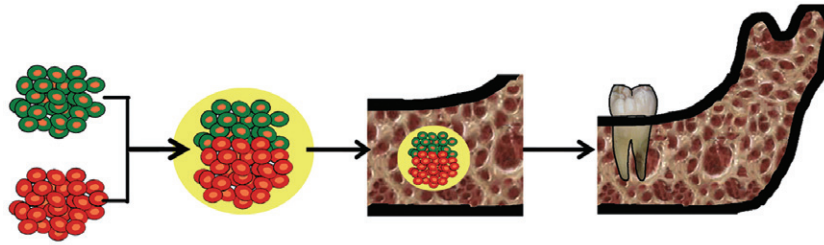


Figure 22.2 Bioengineered organ germ method. Dental epithelial and mesenchymal cells are injected side by side into a collagen drop at a high density. The resulting bioengineered tooth germ can be transplanted into the jawbone for further development. Green indicates epithelial tissue; red indicates mesenchymal tissue.

epithelial and mesenchymal cells at high density into a collagen gel drop (Nakao *et al.* 2007). Dental epithelial and mesenchymal cells were first harvested separately from dissociated cap stage tooth bud tissues; both cell types then were re-aggregated together using cell compartmentalization at a high cell-seeding density. Morphologically and histologically correct molar and incisor tooth buds were observed after *in vivo* transplantation. The regenerated tooth buds were also shown to develop into functional teeth when implanted in the diastema of adult mice (Ikeda *et al.* 2009). To date, this result is the most promising method for functional whole-tooth regeneration. However, the same limitation exists for this approach—only embryonic cap-staged tooth bud cells exhibited tooth formation. It is still not clear if this method requires the use of embryonic tooth bud cells. If so, an urgent question will be how to generate appropriate human epithelial cell sources.

Cell-homing method for tooth regeneration

In situ tooth regeneration would require biomaterials that release bioactive molecules that could attract stem cells in surrounding tissues or from the circulation, which could be stimulated to regenerate new dental tissues (Karp & Leng Teo 2009). Unlike cell transplantation, this cell-homing approach does not require the addition of cells. Cell-homing methods for tissue regeneration are fairly new in the dental field. In one example, Mao's group filled the pulp chambers and root canals of endodontic treated teeth with growth factors, including vascular endothelial growth factor (VEGF-2), fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), and bone morphogenetic protein-7 (BMP7; Kim *et al.* 2010a). Pretreated human teeth were then subcutaneously transplanted into mice, and pulp-like tissue with erythrocyte-filled blood vessels was observed after three weeks. The same group also manufactured polycaprolactone (PDL) and hydroxyapatite (HA) tooth scaffolds containing stromal-derived factor-1 (SDF1) and bone morphogenetic

protein-7 (BMP7) and implanted them subcutaneously or into the jawbone of rat. Distinct periodontal ligament and new bone, but no dentin or enamel tissues, were regenerated (Kim *et al.* 2010b). To date, no research using this method has successfully demonstrated the ability to induce surrounding cells to differentiate into ameloblasts. Therefore, there are no data supporting the possibility of whole-tooth regeneration using the cell homing method. However, this method offers an alternative method for tooth engineering without regenerating the epithelial-mesenchymal interaction.

Tooth bud–bone hybrid constructs

Most dental tissue-engineering efforts have only demonstrated the regeneration of tooth crown structures. Since the mechanisms regulating tooth root formation and eruption remain unclear, it is difficult to use these signals to facilitate bioengineered tooth root formation and eruption. The fact that bioengineered teeth can be generated using the tooth organ germ method suggests that alveolar bone may play an important role in tooth eruption. A bone–tooth bud model (Figure 22.3) has been tested before, where bioengineered tooth bud scaffolds were combined with bioengineered bone constructs and implanted in rat or pig jaws. Although well-organized dental tissues formed in these *in vivo* jaw implants, close integration of newly formed dental tissues and surrounding bone tissues was observed (Duailibi *et al.* 2008; Abukawa *et al.* 2009; Zhang *et al.* 2009). It is anticipated that further modifications of this model will facilitate tooth regeneration and eruption.

Current problems and possible solutions for successful tooth regeneration

Inefficient blood vessel formation is one of the main issues that restrains the size of the regenerated tissue (Griffith & Naughton 2002). Angiogenesis is required for oxygen and nutrient delivery. Without proper blood vessel formation, necrosis will occur at the center of an

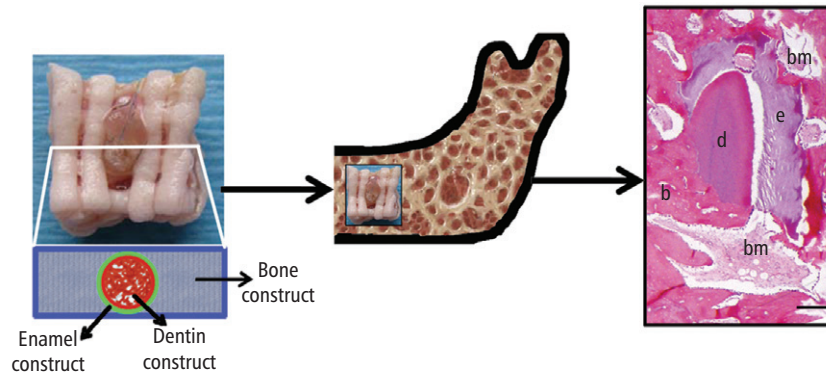


Figure 22.3 Tooth bud–bone hybrid constructs. Hybrid tooth bud–bone constructs were generated from autologous porcine bone marrow stromal cells and dental epithelial and mesenchymal tooth bud cells, respectively, as shown. The hybrid constructs then were transplanted into the same porcine jaw bone. Organized and distinct tooth tissues were generated. However, the regenerated dental tissues were encapsulated in the surrounding newly formed bone. b: bone; bm: bone marrow, d: dentin; and e: enamel.

implant. Noncalcified dental tissues, including tooth bud and dental pulp, are highly vascularized tissues. Inducing the formation of new blood vessels is an important consideration for successful tooth regeneration. One possible solution is to engineer a scaffold that can slowly release angiogenesis stimulate growth factors such as vascular endothelial cell growth factor (VEGF). Designing a scaffold with a degradation rate matching the ingrowths of new tissue would also be helpful.

Another issue is how to identify and isolate appropriate dental stem cell populations. Although the existence of dental stem cells is now well accepted, their exact origin remains unknown. Within the pulp, the migration of mitotic cells suggests that dental pulp cells (DPCs) are from the central part of the pulp (Fitzgerald *et al.* 1990). Currently, dental stem cell isolation methods can achieve only heterogeneous cell populations. Since the ratio of “true” dental stem cells (DSCs) in the cell population is anticipated to be fairly low (i.e., on the order of 0.01%–1%), dental tissue regeneration results will certainly be influenced by the presence of non-DSC types. Fluorescence-activated cell sorting (FACS) is commonly used to obtain more purified stem cell populations. However, no universally accepted stem cell marker has been identified at this time.

There is an additional concern. Ex vivo expansion is necessary to obtain sufficient stem cell populations for dental tissue regeneration. Most existing protocols for cell culture use animal serum, a rich source of nutrients and growth factors, to increase cell proliferation rates. Culturing cells with animal sera, or with other animal products, introduces the potential risk of transmitting animal diseases. The use of autologous serum, or fresh frozen human plasma to replace animal products, is one of the approaches currently being used to solve this

problem. Another possible solution is to develop chemically defined, serum-free media. A recent publication reported that three commercially available serum-free media supported the proliferation and multipotency of human SHEDs and PDLSCs (Tarle *et al.* 2011), although whether these cells have retained their ability to regenerate dental tissues is not yet known.

Conclusion

Although millions of dollars are spent each year on tissue engineering, only a few bioengineered tissue-engineering products have been approved by the US Food and Drug Administration (Fink 2009). Stem cell–based tissue-engineering approaches provide the most promising solutions for dental tissue-engineering strategies, and autologous dental pulp stem cells offer the best cell source, but are not always available. The ability to use iPSCs successfully for dental pulp regenerative therapies could eventually provide a practical alternative cell source.

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Clinical correlate: periodontal regeneration

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Bacterial plaque in a susceptible host is the primary cause of periodontal disease. Clinically, the distinguishing sign of periodontal disease is the loss of soft tissue attachment and bone. Conventional treatment modalities such as nonsurgical and surgical debridement of root surfaces with possible recontouring of the bony architecture often result in healing by repair. Though effective, these therapies are not ideal. Periodontal regeneration, which is the restoration or reconstitution of a lost or injured part (American Academy of Periodontology (AAP) 2001), is the optimal treatment outcome in the management of periodontal disease.

Periodontal regeneration stems from the “compartmentalization” hypothesis proposed by Melcher (1976). He observed that periodontal ligament cells, alveolar bone cells, and perhaps cementoblasts had regenerative capabilities in contrast to epithelial cells, which produced a long junctional epithelium during wound healing. For this reason, the cell type that first populates the periodontal wound site determines if repair or regeneration of the defect occurs. Epithelial cells typically migrate to the wound site at a faster rate compared to periodontal ligament cells, osteoprogenitor cells, or cementoblasts. Therefore, exclusion of epithelial cells will allow other cells with regenerative potential to colonize the defect first, minimizing the potential formation of a long junctional epithelium. This gave rise to the concept of guided tissue regeneration, which is defined as:

Procedures attempting to regenerate lost periodontal structures through differential tissue responses. Barrier techniques are employed in the hope of excluding epithelium and the gingival corium from the root or existing bone surface in the belief that they interfere with regeneration. (AAP 2001)

There are two broad categories of barrier membranes. The most commonly used nonresorbable membranes are the expanded and non-expanded polytetrafluoroethylene membranes. The bioabsorbable membranes include polyglactin-910, polylactic acid, acellular dermal matrix, periosteum, calcium sulfate, and collagen. In general, bioabsorbable membranes are preferred over the nonresorbable membranes because a second surgical procedure to remove the membrane is not required plus they are more forgiving when exposed to the oral environment. However, they lack the rigidity and space maintenance property of the nonresorbable membranes. As a result, bone graft materials are frequently used in combination with bioabsorbable membranes. Various bone grafts such as allografts, xenografts, and alloplasts are available commercially.

In certain clinical situations where patient factors (e.g., immunocompromised medical status) and defect factors (e.g., wide and shallow defect morphology) are unfavorable, the use of tissue engineering can provide the additional boost needed for regeneration of periodontal defects. Tissue engineering is an emerging multidisciplinary field involving biology, medicine, and engineering that aims to improve health and quality of life by restoring, maintaining, or enhancing tissue and organ function. In the field of periodontics, it refers to the repair of alveolar bone, cementum, and PDL. The interplay of four key elements—availability of regenerative cells, presence of appropriate signaling molecules, an adequate blood supply, and a stable scaffold for cellular in-growth—dictates the success of periodontal tissue engineering (Tabata et al. 2005).

Research on local application of bioactive agents such as bone morphogenetic proteins (BMPs), platelet-derived

growth factor (PDGF), enamel matrix proteins (EMDs), platelet rich plasma (PRP), and parathyroid hormone (PTH) in the management of periodontal defects has shown promising results (Giannobile & Somerman 2003). In addition, incorporation of stem cells and gene therapy for periodontal regeneration is a direction for future research to optimize the therapeutic outcomes (Lin *et al.* 2009).

Case presentation

This case report documents a successful application of guided tissue regeneration in the treatment of a periodontal defect. The patient, Mr. C., presented to the Graduate Periodontics Clinic at the University of Michigan School of Dentistry for a periodontal evaluation. He was previously told that he had periodontal disease and he indicated that he desired treatment. He had no other chief complaints.

Mr C. is a 41-year-old Caucasian male. He has well-controlled hypertension, hyperlipidemia, hypercholesterolemia, and a synthetic graft that was placed to repair an abdominal aortic aneurysm two years previously. His medical conditions are well controlled with aspirin 81 mg, atenolol 25 mg, gemfibrozil 600 mg, and simvastatin 10 mg. He takes vitamin C and D supplements daily. He has no known drug allergy and no contributory family history. He has smoked for 35 years and is currently in a smoking cessation program. A medical consultation with his physician revealed that the patient required antibiotic prophylaxis, 2 g of Ampicillin stat, one hour prior to dental treatment.

The patient reported a recurrent periodontal abscess on the facial gingiva of the maxillary right central incisor (tooth 8). The lesion occurred twice in the past year and would regress after a course of antibiotics prescribed by his general practitioner.

A comprehensive periodontal examination comprising extraoral, intraoral, and radiographic assessments was completed (Figures 23.1 and 23.2). An oral hygiene evaluation showed that the patient had poor oral home care with generalized moderate plaque accumulation along the gingival margins, evident interproximal calculus, and moderate staining. His full mouth plaque score was 80%, and his full-mouth bleeding score was 65%. Periodontally, he was diagnosed with generalized chronic severe periodontitis. Tooth 8 had a deep probing pocket depth of 6 mm on the mesial surface. The radiographic evaluation showed that tooth 8 (mesial) had 80% vertical bone loss (Figure 23.1A) with 1° of mobility. The tooth was given a questionable prognosis due to amount of bone loss and mobility (Kwok & Caton 2007).

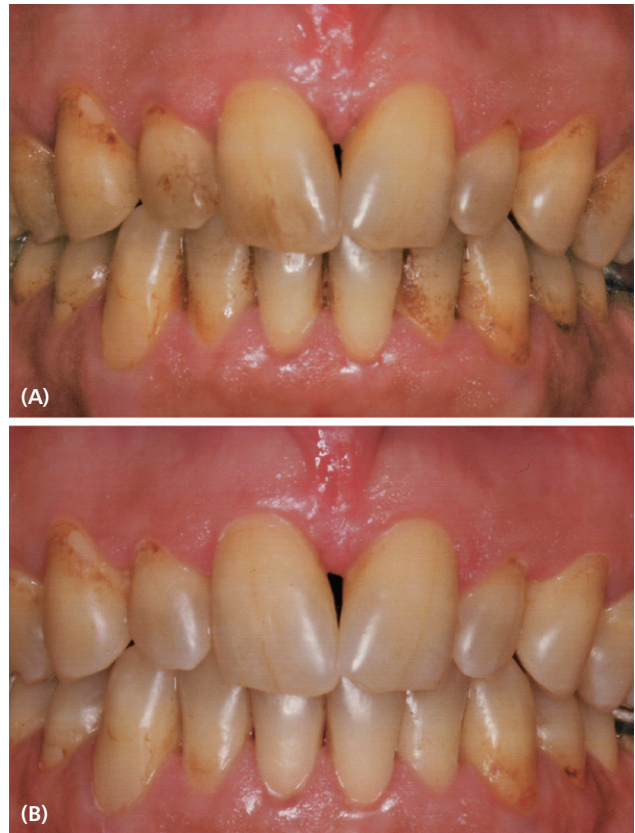


Figure 23.1 A. Initial clinical presentation of tooth 8. B. Clinical presentation of tooth 8 at six weeks post initiation of treatment.

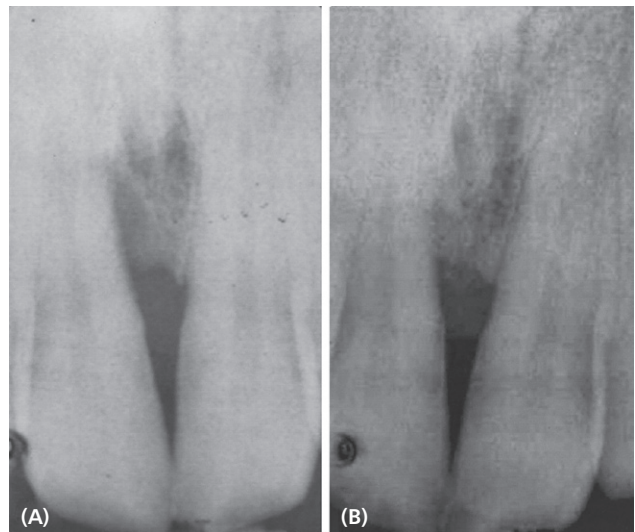


Figure 23.2 A. Initial periapical radiograph of tooth 8 showing the vertical intrabony defect on the mesial surface. B. Periapical radiograph of tooth 8 at five-month re-evaluation visit showing bone fill on the mesial surface.

The initial phase therapy, consisting of scaling and root planing, oral hygiene instructions, and patient education and motivation, was performed. At a six-week re-evaluation, overall improvement in oral hygiene and periodontal health was observed with a reduction in full-mouth bleeding score to 24% and full-mouth plaque score to 25%. However, the probing pocket depth on tooth 8 (mesial) increased to 9 mm (Figure 23.1B). As such, surgical regenerative periodontal therapy was recommended to attempt to regain the lost attachment on tooth 8.

Guided tissue regeneration surgery was performed on tooth 8 (Figures 23.3 and 23.4). A papilla preservation flap was elevated after local anesthesia was administered. The combination of an apical three-wall defect (intra-bony vertical component of 2 mm) and a more coronal two-wall defect (intra-bony vertical component of 2 mm) was thoroughly debrided and degranulated (Figure 23.3A). Cancellous mineralized allograft was gently packed into the intrabony defect, and a collagen barrier

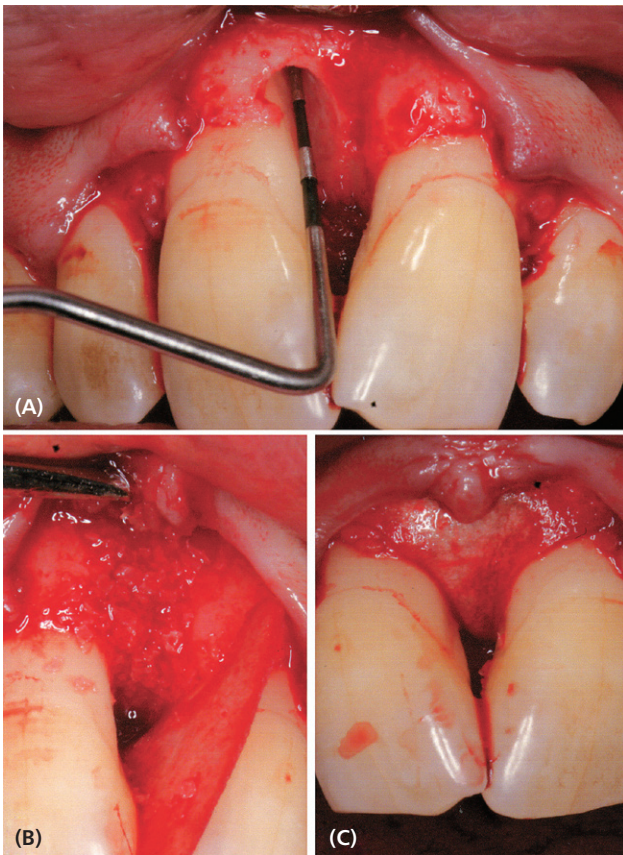


Figure 23.3 A. Clinical presentation of the intrabony defect on the mesial surface of tooth 8 after debridement. B. Clinical presentation of the mineralized bone allograft placed into the intrabony defect. C. Clinical presentation after collagen membrane was placed over the bone allograft.

membrane was placed over the defect (Figure 23.3B). The flaps were re-approximated and sutured securely with an absorbable suture.

Postsurgical healing occurred uneventfully, and at the five-month re-evaluation visit, regeneration in terms of bone fill was seen radiographically on the mesial surface of tooth 8 (Figure 23.2B). Clinical regeneration, in terms of probing pocket depth and clinical attachment level, was also observed (Figure 23.5). The patient is currently on a three-month periodontal maintenance program.

Discussion

Considering clinical and radiographic treatment end points such as clinical attachment level gain, probing pocket depth reduction, radiographic bone fill, tooth survival, and periodontal health, the guided tissue regeneration treatment provided for tooth 8 was a success. Interaction between patient, defect, or other factors played an important role in achieving this outcome.

In this clinical scenario, there were several favorable factors that geared the treatment outcome toward success. The defect was a combination of three-wall (apical) and two-wall (coronal) intrabony defect morphology thus presenting as a contained defect with adequate bony walls to support the influx of blood vessels, angiogenic factors (e.g., PDGF) and cells with regenerative potential (e.g., periodontal ligament cells), and osteoprogenitor cells into the defect, promoting periodontal regeneration. In addition, the total defect depth was 4 mm with a radiographic defect angle of 22.5°, an indication of a narrow and deep defect that was shown to be favorable for regeneration (Cortellini & Tonetti 2000). Mr. C. also maintained impeccable oral hygiene, especially in the maxillary anterior teeth, which was advantageous to the treatment provided (Cortellini & Tonetti 2000). Lastly, Mr. C. had a thick gingival biotype, which was essential in maintaining primary wound closure throughout the healing period, which provided a stable and protected environment for periodontal regeneration to take place.

Smoking was the main unfavorable factor in this clinical scenario. Studies on guided tissue regeneration have consistently showed that smoking affects angiogenesis to the wound site and induces wound exposure, thereby contributing to failure of the procedure (Cortellini & Tonetti 2000). However, with the presence of numerous positive factors and the patient's self-motivation to stop smoking, the adverse effects of smoking were negligible in this case.

Success of regenerative therapies in the treatment of periodontal defects has been well documented by several systematic reviews. Through a meta-analysis, Reynolds

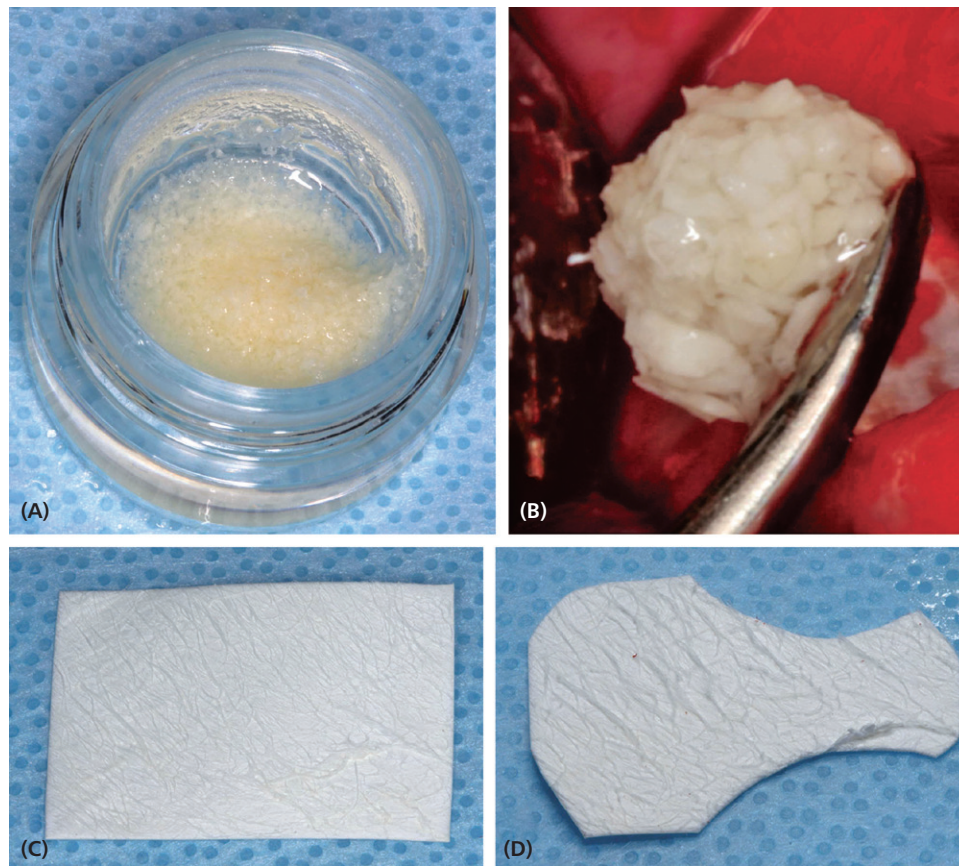


Figure 23.4 A. Mineralized cancellous bone allograft in a vial. B. Mineralized cancellous bone allograft prior to placement into defect. C. Collagen membrane. D. Collagen membrane trimmed to fit the defect.



Figure 23.5 Clinical presentation of tooth 8 at five-month re-evaluation visit.

and colleagues (2003) concluded that the use of bone grafts with barrier membranes had greater clinical attachment level gain and probing pocket depth reduction compared to using bone grafts alone. In addition, new attachment was observed in sites treated with guided tissue regeneration (Reynolds *et al.* 2003). On a similar

note, Laurell and colleagues (1998) found that guided tissue regeneration resulted in significant mean clinical attachment level gain of 4.2 mm and mean bone fill of 3.2 mm compared to both bone graft alone or open flap debridement. A recent *Cochran Collaboration* database systematic review showed that guided tissue regeneration produced a significant improvement in clinical attachment level gain, probing pocket depth reduction, prevention of gingival recession, and hard tissue probing at surgical re-entry when compared to open flap debridement (Needleman *et al.* 2006). Indeed, guided tissue regeneration has proven to be an effective therapeutic modality in the management of periodontal defects. However, it is prudent to note that success of the guided tissue regeneration procedure is largely dependent on careful case selection (Cortellini & Tonetti 2000).

Future research in the field of bioactive agents, stem cells, and gene therapy in periodontal tissue engineering opens a new outlook in the management of a periodontal defect. This not only eliminates problems associated with guided tissue regeneration such as membrane exposure leading to a reduction in attachment gain, but also provides an avenue for optimizing wound healing

and more conservative flap designs, thus improving patient comfort and acceptance.

Summary

Traditional periodontal therapies result in healing by repair. However, the most desirable treatment outcome is periodontal regeneration. It was found that exclusion of the apical migration of epithelial cells would facilitate cells (e.g., periodontal ligament cells, bone cells, and/or cementoblasts) with regenerative potential to repopulate the periodontal defect thus promoting periodontal regeneration instead of repair. With the correct case selection, guided tissue regeneration can be an effective treatment for the management of a periodontal defect.

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Clinical correlate: natural tooth regeneration

Gary E. Heyamoto

It has generally been accepted that severe intradentinal fractures (nonreduced breaks) cannot heal spontaneously by dentinal regeneration. Although many studies have demonstrated dentinal bridging is possible using Ca(OH)₂ (Accorinte *et al.* 2008) or mineral trioxide aggregate (MTA; Conti *et al.* 2009) on carious pulpal exposures, no predictable treatment for severely fractured dentin exists. However, reported cases of unpredictable and possibly anecdotal repairs do exist. The case reported here was documented over several years and offers convincing evidence of spontaneous biological dentinal repair or dentinal regeneration of a horizontally nonreduced fractured molar without the aid of Ca(OH)₂ or MTA. Tooth vitality and function has remained intact for more than 12 years post trauma.

Case presentation

E.B., a healthy Caucasian 11-year-old boy, first presented to the private general practice in 1998, one week after being hit in the face by a baseball. He and his mother were seeking a second opinion for his inability to masticate without discomfort on his left side.

Clinical findings included no symptoms to palpation along the lingual or buccal of teeth 19, K, L, or M. There also was no sensitivity to hot/cold; however, E.B. did feel discomfort upon clenching a cotton roll between teeth 14 and 19. A periapical radiograph was taken of the area of tooth 19 (Figure 24.1A). There was no evidence of pulpal necrosis, but a horizontal fracture emanating from the mesial cervical area was quite evident. A preliminary diagnosis of an unrestorable fracture was made, and arrangements were made for E.B. to see an oral surgeon.

It was unclear if the sensitivity upon pressure was due to the fractured tooth or from a vertical fracture of the mandible discovered by the oral surgeon. It was the surgeon's suggestion that the fractured tooth be extracted after the hairline fracture of the mandible has healed. It was further decided to allow the mandible to heal without intervention since more than a week had passed, healing had started and the fracture was already reduced and required no fixation.

Monitoring E.B.'s comfort level over the next six weeks showed decreased discomfort to chewing with no signs of abscess. The tooth remained symptom free, testing vital with normal function. The decision was made to forego extraction until the discomfort returned or signs of abscess appeared.

Orthodontic advancement of tooth 18 with subsequent advancement of tooth 17 was considered along with future plans for a dental implant for tooth 19. E.B. never had any negative sequelae.

During the next 12 years, periodic periapical radiographs were taken (Figure 24.1B–E). The radiographic series shows the slow elimination of the fracture with what appears to be dentinal regeneration or repair. The tooth still tests vital and shows no radiographic evidence of pulpal necrosis. The impacted third molar (tooth 17) was extracted by an oral surgeon in August 2005, with uneventful healing (Figure 24.1C–D).

Discussion

From the initial radiograph, tooth 19 appeared to have sustained a major horizontal fracture that was very close to the pulp. To have the tooth remain vital and the fracture “disappear,” as suggested from subsequent

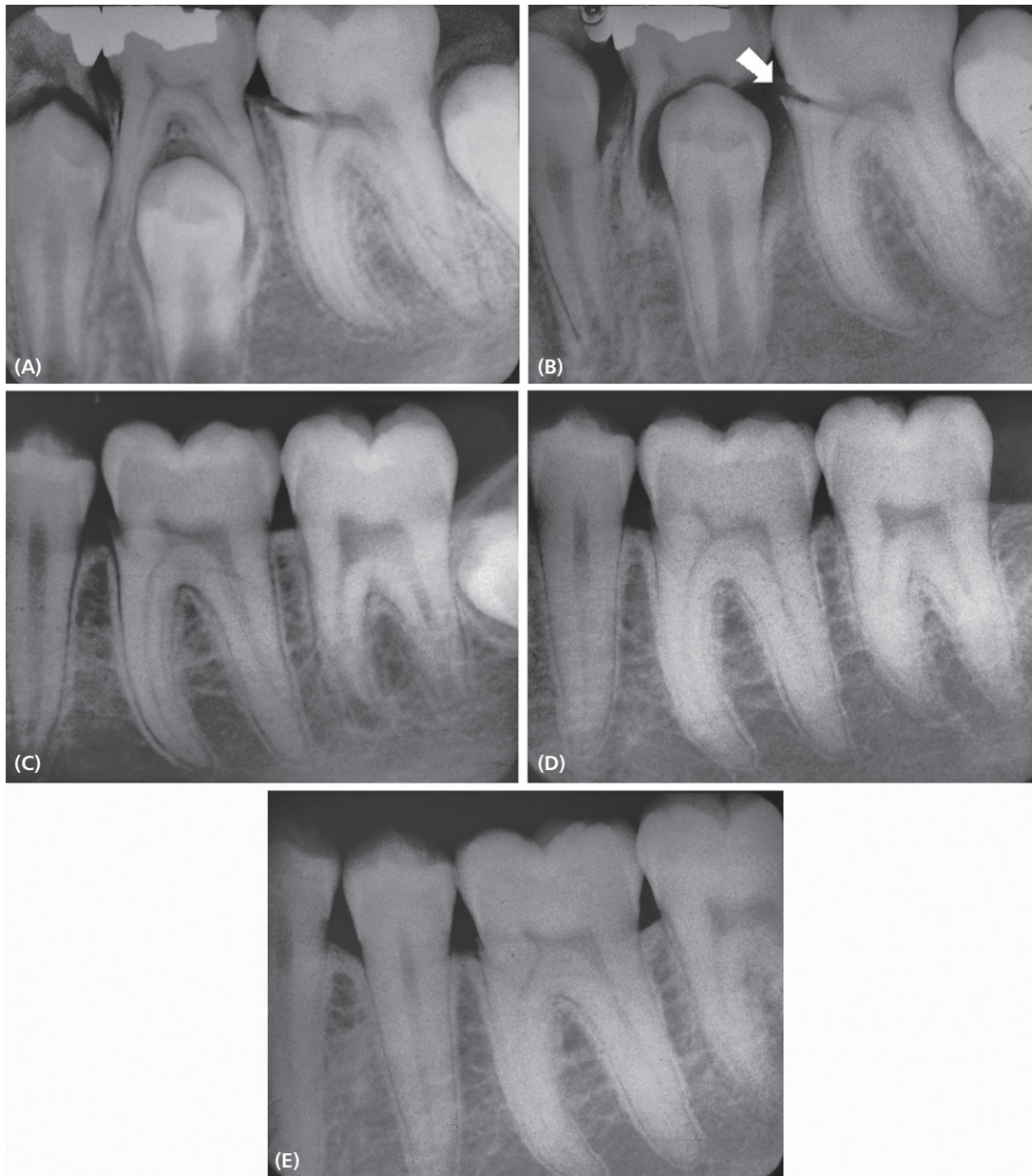


Figure 24.1 Sequential radiographies depicting dentinal repair/regeneration of tooth 19 occurring over a 12-year period. A. An initial radiograph was taken in August 1998; note the horizontal fracture of tooth 19 very close to the pulp. B. Radiograph taken in November 1999; note evidence of dentinal repair in the region closest to the pulp. C. Radiograph taken in April 2001; note the substantial repair of the horizontal fracture, which appears radiographically to be delineated from the normal dentin. D. Radiograph taken in June 2008; note the continued healing, which now makes it difficult to delineate the repair or regeneration region from the normal dentin. E. Radiograph taken in December 2009; healing appears complete with no radiographic change from that seen in D. The radiographs were taken with Gendex GX-770 at 70 kVp 7 mA using AGFA E/F Speed Group 150 3665/5799 film.

follow-up radiographs, some form of dentin alteration must have occurred. This response strongly indicates that some type of dentin regeneration was responsible. No definitive treatment was ever rendered, although the patient was closely monitored with periodic radiographs. The change was not due to iatrogenic influence.

In considering the various explanations for the observed spontaneous healing, it is tempting to focus on nutrients or factors supplied by the follicle region of the erupting premolar (tooth 20) as a logical explanation for this result. As discussed in several basic chapters in this volume and highlighted by studies from Wise's labora-

tory (Yao *et al.* 2008; Wise 2009), it is well established that the follicle region of erupting teeth contains factors such as bone morphogenetic proteins and other morphogens, growth factors, and cytokines known to have the capacity to promote mineral tissue formation. Whether or not these factors influenced mineralization directly and/or stimulated the release of factors from cells within the pulp region that have the capacity to promote mineralization remains unknown at this time. However, it does appear to be a logical explanation for the healing observed, especially when one considers a comparable example, for example repair of simple bone fractures, housed in a closed-wound area and surrounded with a rich nutrient, cellular, and vascular bed.

Conclusion

It has generally been accepted that severe intradentin fractures (nonreduced breaks) cannot heal spontaneously by dentinal repair or regeneration. Occasionally, cases such as the one presented in this chapter offer radiographic evidence that suggests the opposite might

be possible. Although not predictable, dentinal regeneration may be a spontaneous event that occurs when certain conditions exist. In this case, the proximity to an erupting tooth may have influenced the results. Future studies targeted at characterizing factors within the erupting pathway may shed light on explaining this phenomenon, as well as inform therapies for treating comparable fractures in the future. These include situations where teeth are fully erupted and thus not exposed to this rich bath of nutrients.

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Clinical correlate: regenerative endodontics in an immature tooth with pulpal necrosis and periapical pathosis

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Regenerative endodontics is a comprehensive term used to describe recently developed clinical protocols to treat patients presenting with immature permanent teeth with pulpal necrosis resulting, typically, from caries or trauma. The objective of the treatment is to induce intracanal mineralized tissue regeneration and completion of root development.

Traumatic injuries cause permanent tooth loss in 7.4% of the population between 6 and 16 years of age (Wriedt *et al.* 2010). The main consequences of dental trauma occur within 5–10 years after the traumatic event and include pulp canal obliteration, pulp necrosis and infection, root development arrest, external inflammatory and replacement root resorption, and marginal bone loss (Andreasen 1989). Treatment of immature nonvital permanent teeth has previously been limited to therapies directed toward achieving apical closure or apexification, followed by conventional root canal treatment. However, these treatment modalities typically involve multiple visits, long-term treatment, and unpredictable results, with a high incidence of root fractures in the event of another trauma due to thin dentinal walls (Cvek 1992). Regenerative endodontic procedures aim to provide an environment whereby continuation of root development can occur with a more favorable long-term outcome for the patient.

Prior to induction of regenerative endodontic procedures, disinfection of the infected root canal system is required. In published case reports, this has been performed by using copious irrigation with an antimicro-

bial solution (e.g., sodium hypochlorite), followed by the placement of an interappointment intracanal medicament. One such medicament, commonly referred to as *triple antibiotic paste*, is composed of equal proportions of the antibiotics ciprofloxacin, metronidazole, and minocycline, which has been previously shown to eradicate bacteria from infected dentin in vitro (Hoshino *et al.* 1996; Sato *et al.* 1996).

In 2001, Iwaya and colleagues published the first case report describing a regenerative endodontic procedure for an immature lower bicuspid with pulpal necrosis and chronic apical abscess. The tooth was initially treated with an intracanal double antibiotic mixture of ciprofloxacin and metronidazole, followed at a second visit by placement of calcium hydroxide (CaOH₂) in the coronal one third of the root canal. After 30 months, there was radiographic evidence of completion of root formation and thickening of the dentinal walls. The authors claimed that the treatment had promoted revascularization, with residual vital tissues at the apical zone repopulating and differentiating to form the new tissue (Iwaya *et al.* 2001). Subsequently, Banchs and Trope (2004) described a “new treatment protocol” employing triple antibiotic paste as an intracanal medicament for the disinfection of an immature lower premolar with pulpal necrosis and chronic apical abscess, followed at a second visit by generation of an intracanal blood clot onto which was placed a coronal plug of mineral trioxide aggregate (MTA). At 24 months, completion of root formation and dentinal wall thickening were evident radiographically.

Since 2006, several case reports and case series have been published in the endodontic literature describing excellent clinical outcomes using regenerative endodontic protocols for immature teeth presenting with pulpal necrosis with and without periapical pathosis. Here we describe a case in which regenerative endodontic therapy induced dentinal wall thickening and completion of root formation in an immature permanent maxillary central incisor with pulp necrosis and symptomatic apical periodontitis that was the long-term result of a traumatic injury.

Case report

A healthy nine-year-old female patient was referred from the emergency clinic to the graduate endodontic clinic at the CES University, School of Dentistry, Medellin, Colombia, for treatment of her upper left central incisor. The patient presented at the emergency clinic complaining of pain associated with the maxillary left central incisor (tooth 9). One month previously, the tooth had

sustained a complicated crown fracture as a result of trauma. The patient had been symptom-free until recently. There was no contributory medical history, history of previous trauma, or known drug allergies. Root canal treatment was initiated at the emergency clinic for tooth 9 with placement of intracanal $\text{Ca}(\text{OH})_2$ paste, and the coronal access cavity was sealed with composite resin.

The patient presented two days later to the graduate endodontic clinic for continuation of treatment. Examination of tooth 9 revealed a nonfluctuant swelling in the buccal area, sensitivity to palpation and percussion, and increased buccolingual mobility (+1 mm) compared to adjacent teeth. The tooth did not respond to cold and electric pulp tests. Radiographs showed incomplete root formation. Periodontal probing depth on the midbuccal aspect was 5 mm. The diagnosis made for tooth 9 was complicated crown fracture with previously initiated root canal treatment and symptomatic apical periodontitis (Figure 25.1A). The patient was given a local anesthesia with 36 mg of 2% lidocaine with 1:80,000

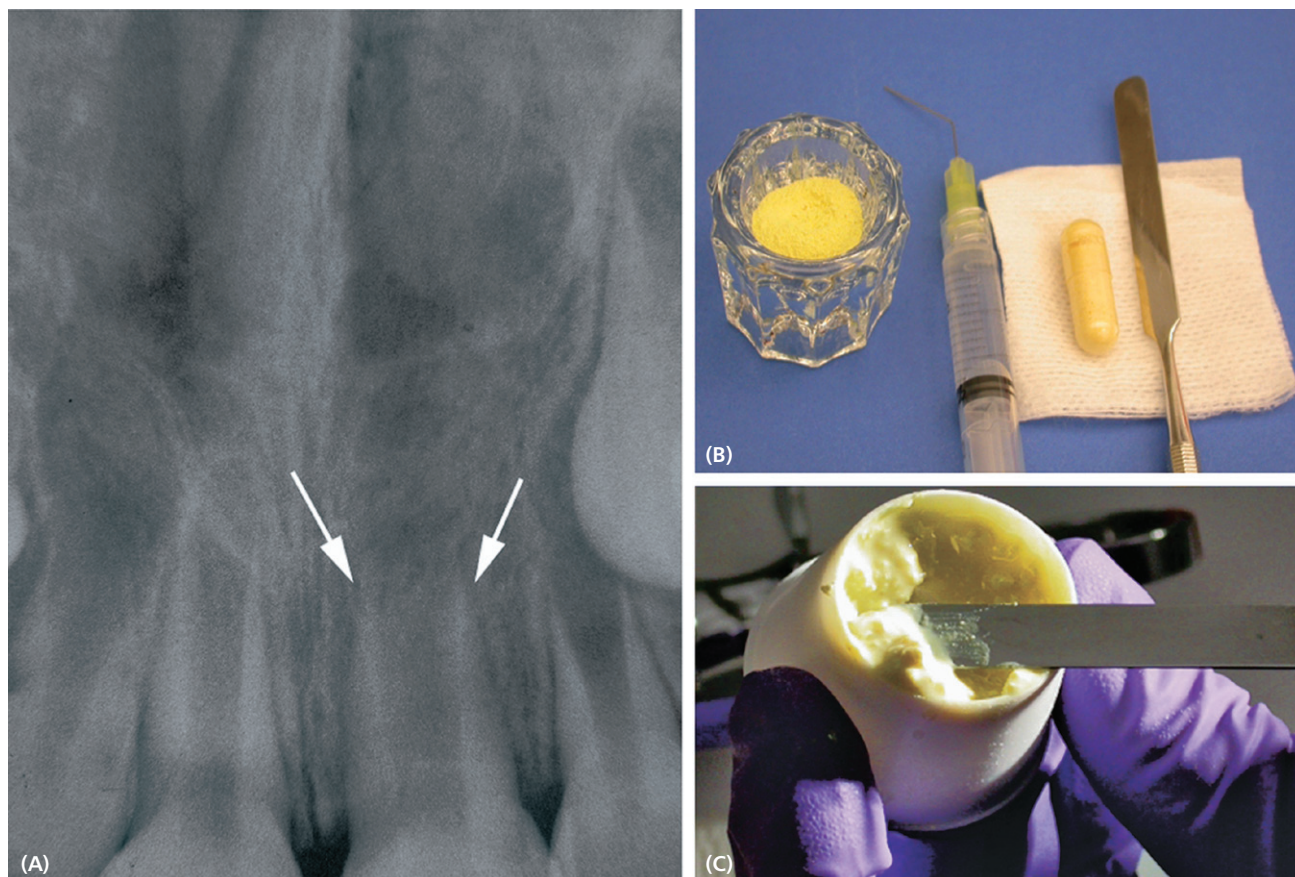


Figure 25.1 A. Maxillary left central incisor with incomplete root formation and open apex (white arrows) one month post trauma and two days following emergency root canal treatment. B. Triple antibiotic powder and sterile distilled water ready to be mixed. C. Triple antibiotic paste mixed to a creamy consistency. (Reprinted with permission from Paniagua, 2010, with permission from CES Odontología.)

epinephrine, the tooth was isolated with a rubber dam, the temporary restoration removed, and the root canal system accessed. The canal was irrigated with 5% sodium hypochlorite (NaOCl) and dried with sterile paper points. No root canal procedure was performed. A triple antibiotic paste was prepared by mixing ciprofloxacin, metronidazole, and minocycline, in a ratio of 5:5:1 respectively, which was mixed with sterile distilled water until the paste had a creamy consistency (Figure 25.1B–C). The paste was introduced into the root canal with a lentulo spiral, and covered with a sterile cotton pellet. The access cavity was sealed with a zinc oxide–zinc sulphate temporary restoration.

At the following appointment one week later, the patient was asymptomatic with no sign of infection. Following administration of local anesthesia and rubber dam isolation, the root canal was accessed, and the triple antibiotic paste removed with copious irrigation of 5% NaOCl. The canal was dried with paper points. Bleeding into the canal was initiated by stimulating the apical tissues with a #80 file introduced into the root canal beyond the apical opening. Over the next 15 minutes, a blood clot was allowed to develop in the root canal from the apex up to a level 5 mm from the cemento-enamel junction. A 3 mm thick layer of white MTA was packed onto the blood clot. A wet cotton pellet was placed onto the MTA, and the access cavity was sealed with Coltosol (Figure 25.2). At the following appointment one week later, the patient was asymptomatic. The tooth was isolated with a rubber dam, and the temporary restoration and cotton pellet were removed. It was noted that the MTA had achieved a hard set. The crown was temporarily restored with glass ionomer cement lining and composite resin Z350 (3M Espe).

At seven months, the patient had no clinical signs of infection. Tooth 9 responded normally to percussion and palpation. Periodontal probing depths were within normal limits. Mild coronal discoloration was noted. Radiographic examination revealed formation of a dentinal bridge and thickening of the dentinal walls of the root apical to the MTA, with no indication of periapical pathosis (Figure 25.3). The patient was referred to her general dentist for a permanent coronal restoration.

The patient did not return for further follow-up appointments until 30 months later at which time the tooth was functioning normally. There was no pain to percussion and palpation. Periodontal probing depths were within normal limits. Radiographic examination revealed further thickening of the dentinal walls compared to the initial radiograph and the radiograph taken at the seven-month recall; at 30 months post treatment, root development was complete, and there was an intact lamina dura and a normal periodontal ligament space



Figure 25.2 Radiograph taken one week following placement of triple antibiotic paste. Bleeding was induced from the apical tissues by using a size #80 file to generate bleeding into the root canal to a level 5 mm from the cemento-enamel junction. White MTA was placed on the blood clot. (Reprinted with permission from Paniagua, 2010, with permission from CES Odontología.)

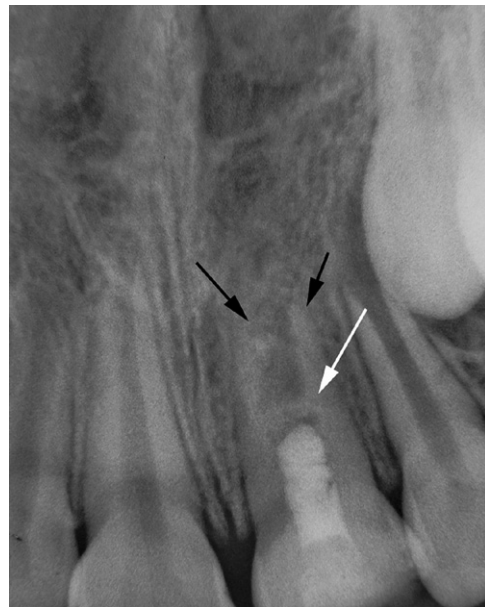


Figure 25.3 Seven-month-recall radiograph showing dentinal bridge formation (white arrow) and increased thickness of the root canal wall apical to the MTA (black arrows). (Reprinted with permission from Paniagua, 2010, with permission from CES Odontología.)

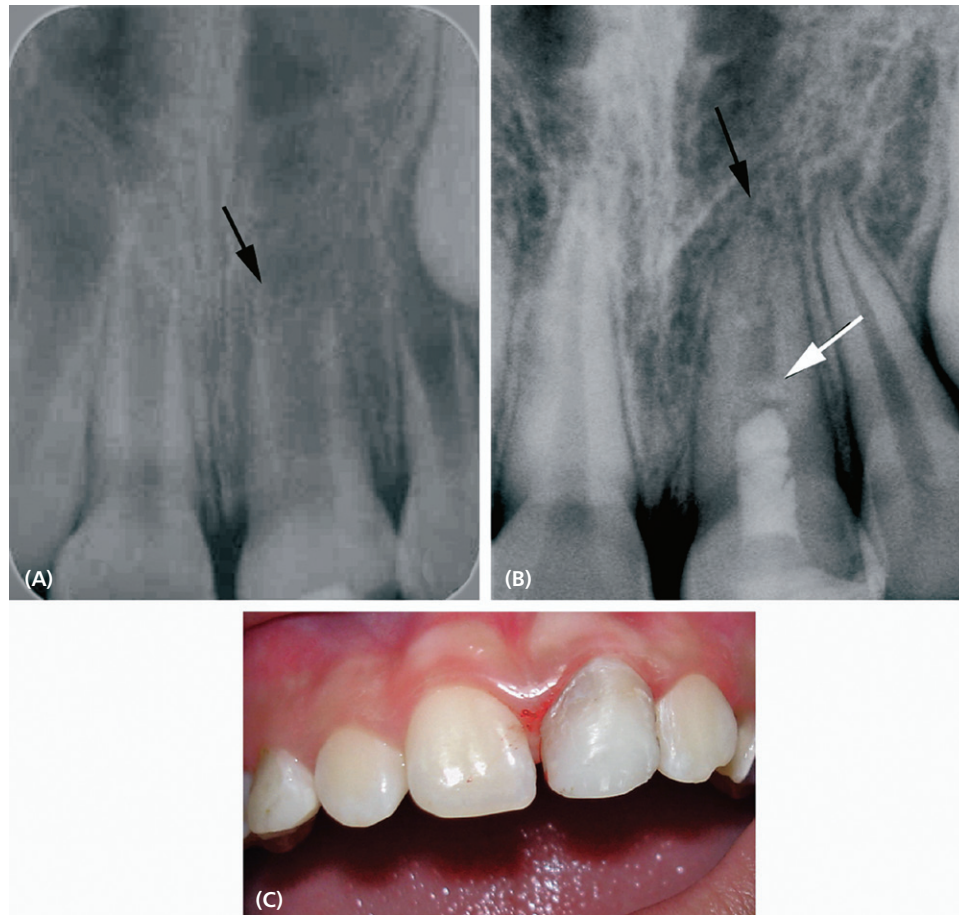


Figure 25.4 Comparison of dentinal wall thickness. A. Radiograph taken at initial presentation. B. Radiograph taken 30 months post treatment showing dentinal bridge formation (white arrow), increased root length, and apical closure (black arrows). C. Moderate discoloration of crown seen 30 months post treatment. (Reprinted with permission from Paniagua, 2010, with permission from CES Odontología.)

(Figures 25.3 and 25.4). The coronal discoloration at 30 months was similar to that seen at seven months.

Discussion

In the case presented in this chapter, regenerative endodontic procedures were used to successfully treat an immature permanent tooth that had sustained a traumatic injury with subsequent development of pulpal necrosis and periapical pathosis. After seven months of treatment, a dentinal bridge and thickening of the dentinal walls with progression of root development were observed radiographically, and at 30 months post treatment, root development was complete.

The American Association of Endodontics (AAE) proposed the term “regenerative endodontics” to describe the non-invasive endodontic therapy of immature teeth that have lost their natural ability to complete root formation as a result of pulpal necrosis subsequent to

carious injury or trauma (Huang 2008). In regenerative medicine the aim is to replace or regenerate human cells, tissues, or organs in order to restore or establish normal function that in many clinical situations could be achieved only by organ transplantation. The two key elements for tissue regeneration are stem cells and growth factors, which, when associated within a matrix or scaffold, are induced to proliferate, migrate, and differentiate into new functional tissue (Langer & Vacanti 1993).

One of the first events needed to initiate regeneration is revascularization, with the subsequent cascade of events inducing cell migration, proliferation, and differentiation. In the case described here, the patient presented with pulpal necrosis. Therefore, the stem cells could migrate from viable remaining dental apical papilla, periodontal ligament, or bone. In addition, these cells could be activated by the growth factors released from platelets within the blood clot. These growth factors

include platelet-derived growth factors (PDGF $\alpha\alpha$, $\beta\beta$, $\alpha\beta$), transforming growth factor beta (TGF β_1 , β_2), vascular endothelial growth factor (VEGF), and epithelial growth factor (EGF) that can induce angiogenesis and cell proliferation. The clot is mainly formed by fibrin, fibronectin, and vitronectin, important cell adhesion molecules required for cell migration (Marx 2001). Together, these components provide the growth factors and the scaffold needed for stem cells to differentiate and regenerate the dental tissues for completion of root formation or apexogenesis. Presently, there is no definitive information on the type of tissues formed. However, a recent histological investigation in dogs described the newly formed tissue as a cementum-like, which the authors named as “intracanal cementum” (Wang *et al.* 2010).

Triple antibiotic paste and MTA were the materials used for this case. However, similar results have been reported with Ca(OH) $_2$ or formocresol as intracanal medicaments, and with Ca(OH) $_2$ or MTA as the material placed onto the induced blood clot. In a recent retrospective analysis, immature teeth showed a significantly greater increase in root length and root wall thickness when treated with triple antibiotic paste and MTA when compared to other materials (Bose *et al.* 2009).

Tooth discoloration attributed to minocycline has been reported following the placement of triple antibiotic paste (Kim *et al.* 2010). In the present case, moderate discoloration of the cervical third of the crown was observed (Figure 25.4C). The discoloration might be avoidable by sealing the dentinal walls of the access cavity with flowable composite prior to placement of the triple antibiotic paste (Reynolds *et al.* 2009). Other approaches to addressing tooth discoloration include eliminating minocycline from the mixture and using walking bleach procedures after completion of the treatment.

At this time and in the absence of randomized clinical trials, the evidence for specific regenerative endodontic protocols that might be adopted clinically is limited to case reports. Regardless, this case along with previous reports clearly demonstrates that immature permanent teeth can respond favorably to conservative and biologically based endodontic therapy. It is feasible that in the future regenerative endodontic protocols will provide a predictable, conservative, and biologically focused treatment option for a broader range of patients requiring endodontic treatment. The results obtained from these clinical cases can serve as a model for future pulp tissue engineering research. Current research is focused on identifying the optimal combination of stem cells, growth factors, and scaffolds to regenerate a functional dentin-pulp complex (Nör 2006; Hargreaves *et al.* 2008).

Summary

The goal of regenerative endodontics in immature permanent teeth with necrotic pulps is to induce a functional dentin-pulp complex with subsequent intracanal mineralized tissue regeneration and completion of root development. A clinical endodontic protocol first promulgated by Iwaya and colleagues (2001) was used to treat an immature permanent tooth with necrosis to induce revascularization and regeneration in a nine-year-old patient. The root canal was disinfected with sodium hypochlorite irrigation followed by placement of an intracanal triple antibiotic paste for one week. An intracanal blood clot was then induced onto which white mineral trioxide aggregate (MTA) was placed. The crown was restored with composite resin. At seven months post treatment, a dentinal bridge and initial thickening of the canal walls were observed. By 30 months post treatment, completion of root development was evident.

Regenerative endodontic procedures may provide a conservative and biologically focused treatment option for selected patients requiring endodontic treatment of immature teeth. Ongoing research should provide a clearer understanding of the mechanisms behind these processes and the optimal clinical application of multiple new therapies.

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SECTION 3

Bones and teeth

Bone and tooth interface: periodontal ligament

P. Mark Bartold

The periodontal ligament is truly a unique and multifunctional connective tissue (Beertsen *et al.* 1997). This tissue is located between the cementum coating of the tooth root and the alveolar bone lining the tooth socket of the maxilla and mandible. It is a specialized connective tissue that fulfills many functions including support for teeth, sensory input, and protection. It also harbors the necessary elements for tissue regeneration. As with all soft connective tissues, the periodontal ligament is composed of both fibrous and nonfibrous elements in addition to nerves, blood vessels, and growth factors.

Development of the periodontal ligament

Prior to tooth eruption, the periodontal ligament begins to develop when tooth root formation commences. This happens at the same time that Hertwig's epithelial root sheath forms and initiates root formation (Figure 26.1). At this time, the dental follicle cells, which are located between the alveolar bone and the epithelial root sheath, are composed of two different mesenchymal cells originating from the dental follicle proper and the perifollicular mesenchyme (Cho & Garant 2000). The dental follicle proper is seen histologically as a band of cells overlying the dental papilla and the outer surface of the outer enamel organ. The perifollicular tissue is a more loosely arranged tissue comprising cells and collagen fibers layered between the dental follicle proper and the developing alveolar bone (Figure 26.1). Thus the cells of the dental follicle form the interface between cementum and periodontal ligament, while the cells of the perifollicular mesenchyme form the interface between periodontal ligament and alveolar bone. The cells derived from the

dental follicle are responsible for the formation of tooth root cementum, periodontal ligament, and alveolar bone. While these three tissues are anatomically distinct, they are clearly united by a common developmental ancestor.

The development of the periodontal ligament differs between the primary and secondary dentition. For the primary dentition (and those teeth that do not have a precursor), the tooth erupts through recently deposited alveolar bone. On the other hand, secondary teeth develop within a bony crypt lingual to the primary teeth, and alveolar bone must be deposited on the surface of the crypt prior to development of periodontal ligament fibers. With the deposition of alveolar bone, periodontal ligament formation proceeds during both the pre-eruptive phases and eruptive phases in a process similar to that of the primary teeth.

Formation of the periodontal ligament closely follows root formation. Fibroblasts deposit collagen fibers, termed *fringe fibers*, that are subsequently arranged into more tightly packed bundles by cementoblasts on the root surface. These lead to the formation of acellular extrinsic fiber cementum. In a similar manner, collagen fibers become inserted into the alveolar bone through interplay between fibroblasts and osteoblasts. The fibers that are inserted into cementum and bone are termed *Sharpey's fibers*. The collagen fibers eventually span the full width of the periodontal ligament forming principal fiber bundles. While the fibers are tightly packed near the cementum and bone interfaces, they are less tightly packed in the middle third of the ligament that is termed the *intermediate plexus*.

The orientation of the principal fiber bundles differs before and after tooth eruption. The initial orientation

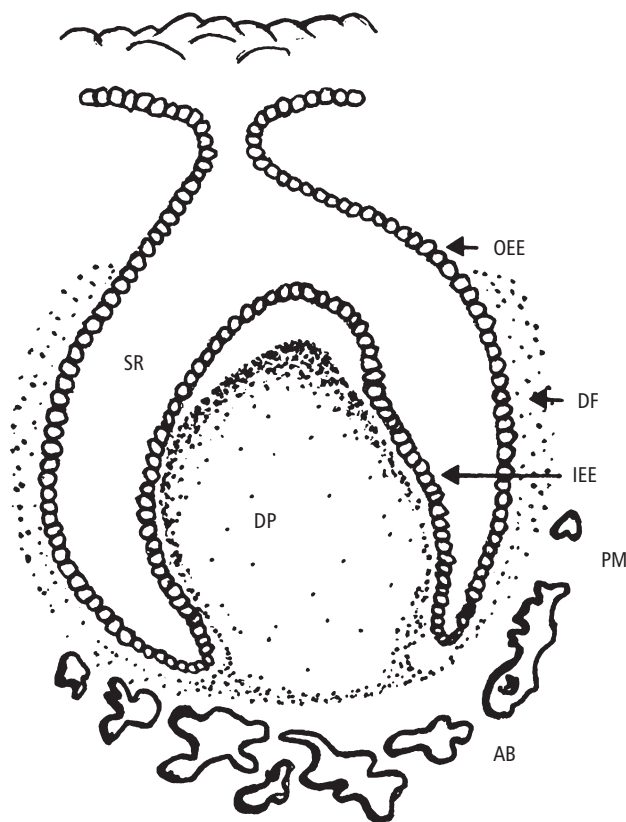


Figure 26.1 Schematic representation of the developing periodontium. The periodontal tissues arise from the dental follicle, developing tooth root, and alveolar bone. Abbreviations: SR: stellate reticulum; DP: dental papilla; AB: alveolar bone; PM: perifollicular mesenchyme; IEE: inner enamel epithelium; DF: dental follicle; and OEE: outer enamel epithelium. (Reprinted from Bartold & Narayanan, 1998, with permission from Quintessence Publishing.)

of the principal fibers of the developing periodontal ligament is in a coronal direction from cementum toward the alveolar bone. This process progresses from the cementoenamel junction to the root apex, thus the first fibers to be formed become the dentogingival fibers and the transeptal fibers of the gingival. Subsequent fibers, deposited below the cementoenamel junction, form the periodontal ligament.

When the tooth begins to erupt, the orientation of the periodontal ligament fibers changes, with the periodontal ligament taking its final arrangement once the tooth is fully erupted and in functional occlusion (Grant & Bernick 1972). At this stage, the dentogingival, transeptal, and alveolar crest fibers project from the cementoenamel junction toward the gingival tissues. The fibers immediately below the alveolar crest fibers and within the coronal one-third of the ligament are arranged in a horizontal manner. Within the middle one-third of the ligament, the fibers are oriented obliquely in an occlusal

Table 26.1 Components of the periodontal ligament.

Cells	Fibroblasts Endothelial cells Nerves Osteoblasts Cementoblasts Mesenchymal stem cells
Fibrous	Collagens Elastin
Nonfibrous	Proteoglycans Fibronectin Tenascin Laminin Periostin Osteonectin Bone sialoprotein Hyaluronan
Growth factors	Transforming growth factors Platelet-derived growth factors Fibroblast growth factors
Lipids	
Water	
Minerals	

direction from the cementum toward the alveolar bone. In the apical one-third, the fibers are also arranged obliquely but running in an apical direction away from the root surface and toward the alveolar bone.

General structure and composition of the periodontal ligament

Since the periodontal ligament is a connective tissue, it is composed of cells embedded within an extracellular matrix of collagenous and noncollagenous proteins, growth factors, minerals, lipids, and water (Table 26.1 and Figure 26.2). The principal cells found within the periodontal ligament are (1) fibroblasts, (2) osteoblasts, (3) cementoblasts, (4) endothelial cells associated with the vasculature, (5) epithelial cell rests of Malassez, and (6) cells associated with the sensory system. The periodontal ligament also contains undifferentiated stem cells that have the potential to differentiate into cementoblasts, fibroblasts, or osteoblasts.

The principal function of the periodontal ligament is to support teeth in their sockets and to withstand the considerable forces resulting from mastication. Another important function of the periodontal ligament is to act as a sensory receptor that permits correct alignment of the jaws during normal function. It also protects the ligament itself from excessive forces. The periodontal

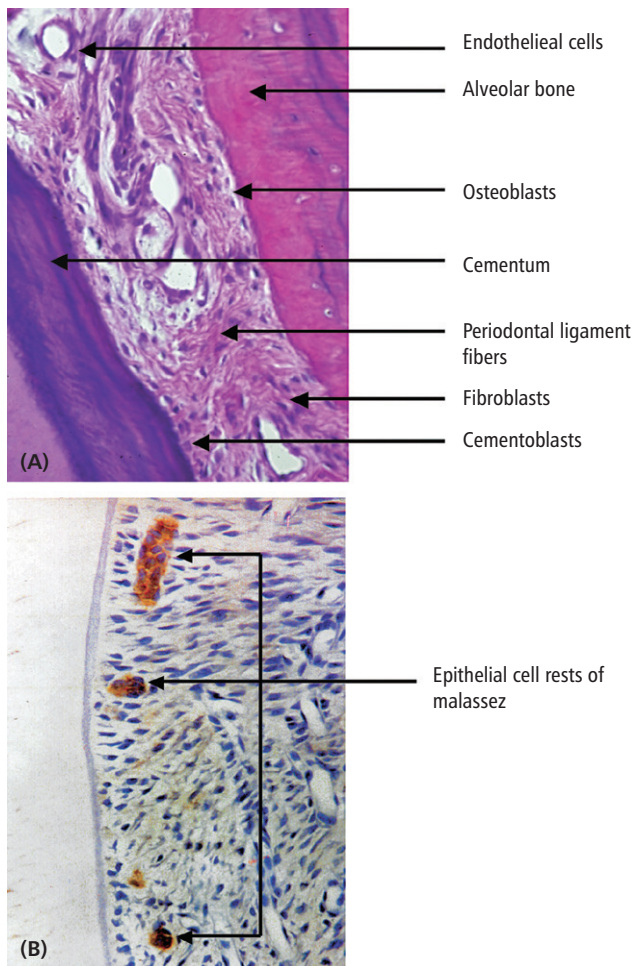


Figure 26.2 Histological features of the human periodontal ligament. A. Section stained with hematoxylin and eosin (H&E) illustrating the cellular distribution throughout the periodontium. B. Epithelial cell rests of Malassez in rat periodontal ligament stained with anti-E11 antibody.

ligament ranges in width from 0.15 mm to 0.38 mm, although this can vary depending on the amount of occlusal forces the tooth withstands.

Collagens of the periodontal ligament

The principal collagen types in the periodontal ligament are the fibrous collagens type I and type III. These are arranged in distinct bundles that maintain the general architecture of the ligament and are generally arranged to withstand the functional forces of occlusion and mastication. In addition to type I and II collagens, a number of other collagen types have been identified in the periodontal ligament (Bartold & Narayanan 1998). Type V collagen has been identified in the periodontal ligament, and this collagen is believed to be located within the core of the major collagen fibril bundles or between the fibrils. Type IV collagen is located within the basement

membrane of blood vessels, and a number of other minor collagens (types VI and XII) contribute to the fibrous network of the periodontal ligament. Another minor collagen (type XIV, undulin) has been associated with the major collagen fibrils of the periodontal ligament and may play a role in the flexible organization of collagen fibrils.

Elastin

Elastin, as its name suggests, provides a degree of elasticity to various connective tissues. It is formed by the deposition of tropoelastin onto microfibrils that are composed of fibrillins, microfibril-associated glycoproteins (MAGPs), vitronectin, fibronectin, proteoglycans, and amyloid (Kielty *et al.* 2002). The elastic fiber system is made up of a series of three fiber types: oxytalan, elaunin, and elastic fibers. Each of these fibers coaggregates with MAGPs to form increasing amounts of elastin. Mature elastic fibers are composed of a central core of elastin surrounded by microfibrils. Oxytalan fibers are a collection of microfibrils minus the amorphous elastin. Elaunin fibers are composed of elastin deposited between microfibrils (oxytalan).

Early histochemical analysis of the periodontal ligament demonstrated the presence of a group of fibers oriented in an apicocoronal direction. Originally identified as oxytalan fibers, these fibers are now generally recognized to be pre-elastic fibers or microfibrils. Recent histochemical analysis of the rat molar periodontal ligament has demonstrated that most of the elastin system within the periodontal ligament is composed of elastin-free microfibrils (oxytalan). Elastin-like fibers around blood vessels in the apical portion of the ligament have been identified as elaunin fibers. No mature elastic fibers have been identified in the periodontal ligament (Sugawara *et al.* 2010).

Proteoglycans

Proteoglycans are composed of a central core protein covalently attached to a variable number of carbohydrate (glycosaminoglycan) side chains. There are two major types of proteoglycans found in the periodontal ligament that have been classified as being matrix-organizing macromolecules or cell surface glycoproteins (Bartold & Narayanan 1998). The matrix-organizing proteoglycans have been further classified as either large aggregating proteoglycans (versican) or small leucine-rich proteoglycans (decorin, biglycan, lumican, and fibromodulin). Versican, which has the capacity to form large hydrated macromolecular aggregates with hyaluronan, has been located throughout the extracellular matrix of the periodontal ligament. This property may be associated in regulating rehydration of the

periodontal ligament following compressive forces placed on it during mastication. The small leucine-rich proteoglycans, decorin, lumican, and fibromodulin, have been found to co-localize with collagen fibril bundles and, in a coordinated manner, regulate collagen fibrillogenesis and fibril bundle formation in the periodontal ligament (Matheson *et al.* 2005). Biglycan is a relatively minor proteoglycan of the periodontal ligament and is expressed on the cell surface pericellularly, and sometimes within the extracellular matrices of a range of specialized cell types within the organ. Biglycan has been proposed to play a role in regulating mineral deposition (Waddington *et al.* 2003), but its precise role in the periodontal ligament is yet to be fully elucidated.

Other nonfibrous proteins of the periodontal ligament

A large number of nonfibrous proteins are found in the periodontium (Table 26.1). Most of these demonstrate distinct and unique distributions and functions throughout the extracellular matrix of the periodontal ligament.

Tenascin is a large oligomeric glycoprotein found between loosely packed collagen fibrils, whereas fibronectin tends to surround individual collagen fibrils (Zhang *et al.* 1993). Due to their localization to cementum and alveolar bone surfaces, tenascin and fibronectin have been proposed to be involved in tissue mineralization. In addition, tenascin has been implicated in responses of the periodontal ligament to mechanical stress (Lukinmaa *et al.* 1991).

Laminins are a group of large multidomain glycoproteins that are composed of three distinct polypeptide chains and are important structural elements of basement membranes. Laminin is also an important extracellular matrix molecules associated with cell attachment, proliferation, and migration. The most thoroughly studied laminin is laminin-1. This is characteristically located at the basement membrane of the junctional epithelium and endothelium of blood vessels of the periodontium.

A key molecule in assisting the absorption and distribution of forces within the periodontal ligament is periostin (Rios *et al.* 2008). This molecule is primarily expressed in the periodontal ligament and is necessary for periodontal homeostasis during occlusal function and inflammation. Periostin influences cell behavior as well as collagen fibrillogenesis and, therefore, exerts control over the structural and functional properties of the periodontal ligament in both health and disease. In mice lacking periostin, alveolar bone and the periodontal ligament dramatically deteriorate following tooth eruption (Rios *et al.* 2008).

A number of cell adhesion proteins and integrins are expressed in the periodontal ligament (Steffensen *et al.* 1992). As detailed above, the cell attachment proteins such as vitronectin, fibronectin, tenascin, and laminin have been well described. Accordingly, cell surface receptors (integrins) for these matrix components have been studied. For example, the vitronectin receptor (alpha v) and fibronectin receptor alpha subunit (alpha 5) are expressed by periodontal ligament fibroblasts (Steffensen *et al.* 1992).

Arrangement of periodontal ligament fibers

As detailed above, once the tooth is fully erupted and in functional occlusion, the fibers of the periodontal ligament assume a specific arrangement. The five major fiber groups of the periodontal ligament are:

1. The alveolar crest group of fibers attaches to the cementum just below the cemento-enamel junction and runs downward and outward to insert into the crestal portion of the alveolar bone.
2. The horizontal group of fibers is located immediately apical to the alveolar crest group and runs at right angles to the long axis of the tooth from the cementum to the alveolar bone just below the alveolar crest.
3. The oblique group of fibers is the most abundant within the periodontal ligament and runs from the cementum in an oblique direction to insert into the alveolar bone coronally.
4. The apical group of fibers radiates out from around the root apex at the base of the alveolar socket.
5. The interradicular group of fibers is found within the furcation region of multirooted teeth and runs from the cementum of the furcation to the alveolar bone forming the interradicular septum.

Fibroblasts

The fibroblasts of the periodontal ligament are the most abundant cell type. These spindle shaped cells have large amounts of rough endoplasmic reticulum and abundant Golgi complexes consistent with their high rate of extracellular matrix synthesis. Interestingly, these fibroblasts synthesize not only collagen but also phagocytose collagen fibrils and then, through intracellular lysosomal (cathepsins B, L, and N) enzyme activity, degrade the ingested collagen (Svoboda *et al.* 1979). This process is integral in the normal turnover of the periodontal ligament. The fibroblasts in the periodontal ligament have a strong binding affinity for both fibronectin and collagen, and this facilitates their migration through the extracellular matrix. In addition, the adherence of these cells to

collagen is responsible for a great deal of the organization of the matrix. The binding of fibroblasts to collagen is facilitated through the expression of specific cell surface receptors known as integrins that bind to specific amino acid sequences in extracellular matrix proteins. Specifically, RGD (arginine–glycine–aspartic acid) sequences in fibronectin-coating collagen fibers can bind to $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins on the fibroblast cell surface. Since the integrins are transmembrane proteins, they facilitate communication between the extracellular environment and the cytoplasm. In this process, the cytoplasmic domain of an integrin binds to several proteins leading to conformation changes in cell shape through their influence on actin microfilament alignment. Through these processes, the extracellular matrix can indirectly link through to the cytoplasm of the fibroblasts and exert a significant influence on cell shape and behavior.

Epithelial cell rests of Malassez

A unique population of cells derived from Hertwig's epithelial root sheath (HERS) also resides within the periodontal ligament. These cells, termed *epithelial cell rests of Malassez*, are of ectodermal origin, arise from the disaggregation of HERS, and persist throughout adult life in the ligament as a meshed network of interconnected cells found in close proximity to the root surface (Figure 26.2). The epithelial cell rests of Malassez are unusual in that they have the capacity to express proteins associated with both ectodermal and mesenchymal tissues (Rincon *et al.* 2005). The function of these cells is still under debate, but their capacity for ectodermal and mesenchymal transformation would suggest a significant role in the maintenance of tissue homeostasis as well as tissue regeneration.

Cementoblasts and osteoblasts

As discussed earlier, the developing periodontal ligament arises from the dental follicle and, within this process, both cementum and alveolar bone are formed, together with the interconnecting periodontal ligament. Both cementoblasts and osteoblasts are discussed in more detail in this book. Therefore, only a brief discussion of these cells is included here to complete this overview of the cells of the periodontal ligament. Cementoblasts and osteoblasts form cementoid and osteoid, respectively, which subsequently undergo mineralization shortly after they are deposited on the developing root surface or bony crypt wall. With the mineralization process, cementoblasts and osteoblasts become embedded in their own matrix and become cementocytes or osteocytes. During regeneration of the periodontium, new

cementum and alveolar bone are laid down, and this presumably arises from cementoblasts and osteoblasts (or their precursors) residing within the periodontal ligament.

Mesenchymal stem cells of the periodontium

It has been recognized for more than 30 years that undifferentiated stem cells or progenitor cells residing in the periodontal ligament have the capacity to give rise to cementoblasts, osteoblasts, and fibroblasts (Gould *et al.* 1977). Early in vivo studies in mice indicated that a group of progenitor cells, exhibiting some classical features of stem cells, could be identified in the periodontal ligament (McCulloch 1985; McCulloch *et al.* 1987). These cells have been located within the perivascular spaces of the periodontal ligament and nearby endosteal spaces (McCulloch *et al.* 1987; Chen *et al.* 2006; Figure 26.3). However, it has not been until recently that these cells have been identified and cultured (Seo *et al.* 2004). These cells have been termed *periodontal ligament stem cells* (PDLSCs).

In vitro, PDLSCs characteristically give rise to adherent clonogenic clusters that resemble fibroblasts (Figure 26.3). They have the potential to differentiate, under appropriate culture conditions, into adipocytes and osteoblast-, cementoblast-, and neuronal-like cells (Seo *et al.* 2004; Techawattanawisal *et al.* 2007). In addition, when implanted subcutaneously into SCID mice in hydroxyapatite tricalcium phosphate scaffolds, these cells can produce cementum- and periodontal ligament-like tissues in vivo (Seo *et al.* 2004). PDLSCs express a number of cell surface markers associated with bone marrow stromal stem cells, including STRO-1 and CD-146 antigens. This suggests that PDLSCs represent another mesenchymal stem cell-like population (Seo *et al.* 2004). Further work is now focusing on identifying markers uniquely expressed by PDLSCs to discriminate these cells from other types of MSC-like cells identified in dental tissues (Menicanin *et al.* 2010). However, this is likely to be a complex task, as earlier studies have indicated that there is considerable heterogeneity amongst cells of the periodontal tissues with regenerative capacity (Ivanovski *et al.* 2001).

Vasculature of the periodontium

The periodontal ligament is a highly vascularized connective tissue, with vessels arising from branches of the dental, intervalveolar, interradicular, and periosteal arteries. In addition to providing nutrients to the periodontal ligament, the periodontal vasculature is also intimately associated with tooth eruption and tooth support (Edwall & Aars 1995). These vessels, which include both

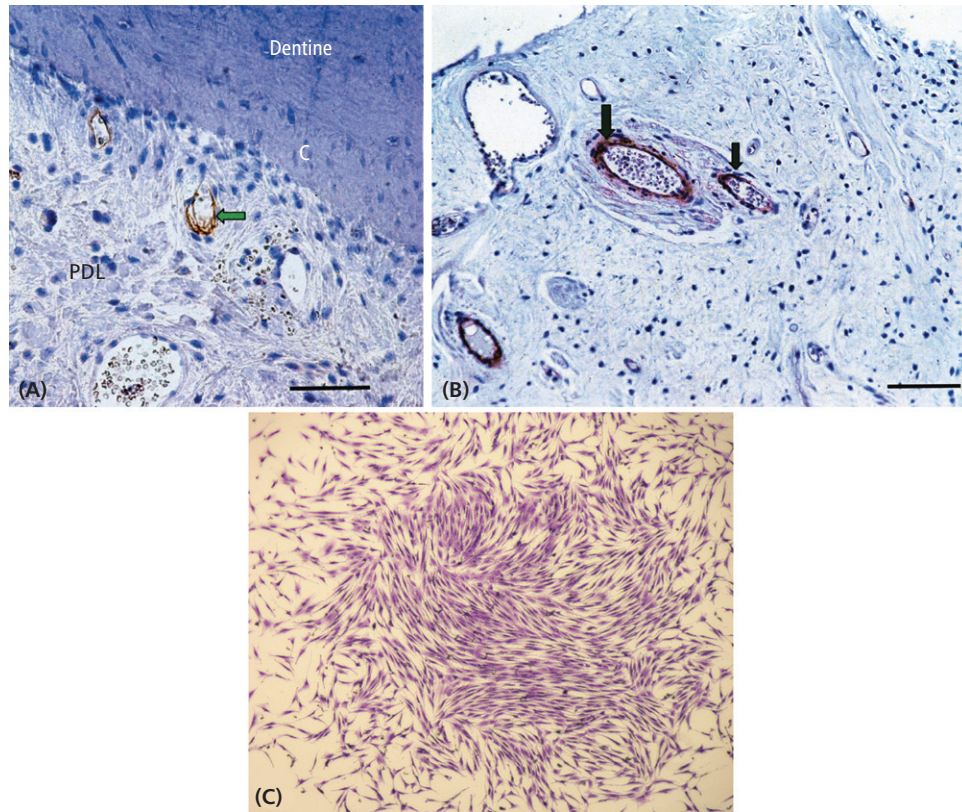


Figure 26.3 Mesenchymal stem cells of the periodontal ligament. A. Staining of periodontal ligament mesenchymal stem cells with anti-STRO-1 antibody demonstrating staining in the paravascular region. B. Staining of periodontal ligament mesenchymal stem cells with anti-CD146 antibody demonstrating staining in the paravascular region. C. Appearance of a colony of mesenchymal stem cells cultured from human periodontal ligament. (A and B are reprinted from Chen *et al.*, 2006, with permission from John Wiley & Sons.)

arterioles and venules, form a basket-like meshwork around the entire tooth root, with the periodontal ligament and gingival vasculature appearing to communicate via a network of anastomoses (Weekes & Sims 1986). This system allows for vascular pulsar flow to occur in different directions (apico-coronal and coronal-apical) throughout the periodontal ligament. These complex arrangements appear to allow the necessary pressure equalizing rebound flow of fluid following masticatory pressure forces that allow rapid rehydration of the ligament following compressive forces.

Innervation of the periodontal ligament

The periodontal ligament is innervated by both myelinated and unmyelinated neurones that allow perception of both pressure and pain (Linden *et al.* 1995). Innervation to the periodontal ligament arises from branches of either the maxillary nerve or the inferior alveolar nerve that enter the periodontal ligament through foramina in the alveolar bone at the apex of the tooth root or along the lateral border of the root. The nerve fibers within the periodontal ligament are found in the outer part of the

ligament space closer to the alveolar bone. In general, the fibers entering the periodontal ligament from the apical foramen run parallel to the root surface in an apico-coronal direction, while those entering from lateral foramina run in both directions. A plexus of nerve fibers develops from both the nerves entering the ligament via the apical region and those entering from the lateral aspects of the alveolar bone. Sympathetic nerves have been identified in the ligament, but no evidence of parasympathetic innervation has been reported.

The receptors in the ligament that respond to forces are termed *mechanoreceptors* (Linden *et al.* 1995). Mechanoreception appears to be associated with A β neurones and pain with the A δ and C fibers. Upon loading of the periodontal ligament, a number of vasoactive neuropeptides are released by sensory nerve endings that interact with blood vessel endothelial cells and fibroblasts. However, by far the most significant function of the periodontal ligament mechanoreceptors is to monitor the forces of mastication and protect the teeth from overload as well as aid in controlling jaw reflexes associated with mastication (Türker *et al.* 2007).

Homeostasis of the periodontal ligament

In adults, normal tissue integrity and turnover of the periodontal ligament are regulated by the fibroblast populations residing within this tissue. These cells normally exist in a steady state where cell renewal, as a result of cell proliferation, is balanced by programmed cell death (apoptosis). The periodontal ligament has a rich source of so-called progenitor or stem cells that have the capacity to give rise to more specialized and differentiated cells responsible for maintenance of the periodontal ligament proper (fibroblasts), cementum (cementoblasts), and alveolar bone (osteoblasts).

Turnover of the periodontal ligament tissues is considered to be amongst the highest of all tissues (Sodek 1976). This allows rapid adaptation to the forces placed upon it during normal function. Turnover and remodeling in response to these forces involve sequential degradation and synthesis of collagens and other extracellular matrix components. While remodeling enables a reorganization of the matrix to adapt to changes in tooth position, turnover is a process of tissue replacement in which the structural arrangement is unaffected. Although degradation of collagen was originally thought to be mediated through the action of collagenase, there is good evidence to suggest that collagen degradation and removal in the periodontal ligament result from phagocytosis by fibroblasts (Everts *et al.* 1996).

After wounding of the periodontal ligament, there is coordinated repopulation of the site by populations of cells with multilineage potential. As a result of specific microenvironment conditions, these cells are directed along specific lineages that, in turn, significantly influence the properties of the newly regenerating tissues. In particular, periodontal ligament fibroblasts can be activated by inflammatory or mechanical stimuli. The activated cells, in turn, can release a number of soluble mediators (cytokines, interleukins, plasminogen activator, and prostaglandin E_2), enzymes (matrix metalloproteinases, and their inhibitors), and extracellular matrix components (collagens, proteoglycans, and elastin). This results in either tissue maintenance or tissue repair and regeneration. Thus, fibroblasts play a central role in homeostasis of the periodontal ligament through their capacity to adapt to their environment and assist in tissue remodeling. They also regulate various cell systems to enable fibrous attachment to both the tooth root and alveolar bone.

Regulation and maintenance of the periodontal ligament space

An important role of the periodontal ligament fibroblasts is to inhibit mineralization and thereby maintain the periodontal ligament as a soft connective tissue span-

ning the space between the tooth root and the alveolar bone. The mechanisms that allow this are still unclear, with both the cells of the ligament and the matrix itself being held responsible for the maintenance of the periodontal ligament width.

Epithelial cell rests of Malassez

An early study noted that ankylosis following reimplantation of teeth did not occur in areas where vital periodontal ligament containing epithelial cell rests of Malassez were present (Løe & Waerhaug 1961). More recently, studies have focused on the cellular and signaling systems that might control this important feature of the periodontal ligament. Periodontal ligament fibroblasts have been considered important since heat treatment of teeth has been noted to induce ankylosis (Line *et al.* 1974). Furthermore, *in vitro* studies have demonstrated that periodontal ligament fibroblasts secrete soluble factors that can inhibit osteogenesis (Lekic & McCulloch 1996).

Prostaglandins

Prostaglandins, specifically PGE_2 and PGF_2 , secreted by periodontal ligament fibroblasts have been reported to inhibit bone formation. These factors may mediate processes through which fibroblasts can modulate osteogenesis at sites between soft and mineralizing connective tissues such as the periodontal ligament (Ogiso *et al.* 1992).

Protein S100A4

Another secreted protein proposed to be involved in maintaining the periodontal ligament is S100A4. It is a member of the S100 calcium-binding protein family. Periodontal ligament cells express the S100A4 mRNA *in vitro*, and this protein has been identified in cryosections of periodontal ligament. S100A4 can inhibit mineralized nodule formation *in vitro* in a concentration-dependent manner (Duarte *et al.* 1999) and is a negative regulator on the expression of osteoblast-related genes (Kato *et al.* 2005). Specifically, S100A4 inhibition by RNAi was noted to upregulate osteoblastic markers such as osteopontin and osteocalcin and the osteoblast-specific transcription factors runt-related transcription factor 2/core binding factor alpha 1 (Runx2/Cbfa1) and Osterix. These results indicate that S100A4 suppresses the expression of osteoblastic genes in periodontal ligament cells and may thus inhibit mineralization in the periodontal ligament (Kato *et al.* 2005).

Periodontal ligament-associated protein

Periodontal ligament-associated protein (PLAP-1), also known as asporin, has been identified as an important

component of the periodontal ligament (Yamada *et al.* 2007). PLAP-1 is similar to the core proteins of the small leucine-rich proteoglycans decorin and biglycan, but it does not contain any glycosaminoglycan chains. Overexpression of PLAP-1 inhibits mineralization by periodontal ligament cells, and knockdown of PLAP-1 mRNA enhances the osteogenic differentiation potential of these cells. These findings suggest that PLAP-1 in the periodontal ligament may prevent cell differentiation and mineralization and contributes to the maintenance of the periodontal ligament tissue.

Matrix GLA protein

Matrix GLA protein, which is a calcium-binding protein that may participate in the organization of bone tissue, is strongly expressed by periodontal ligament cells adjacent to the cementum surface that deposit this protein on the cementum (Hashimoto *et al.* 2001). This protein tends to accumulate at the interface between periodontal ligament and cementum. It has been proposed that one function of matrix GLA protein is to inhibit mineralization. Thus, the expression of this molecule by cells adjacent to the cementum could be one mechanism that prevents mineralization of the periodontal ligament.

Molecular interactions

Interactions between various molecules may also play a role in the maintenance of the periodontal ligament and prevent its mineralization. For example, it has been suggested that maintaining equilibrium between bone sialoprotein and osteopontin levels may be partly responsible for the maintenance of a nonmineralized periodontal ligament (Nanci & Bosshardt 2006).

Extracellular matrix molecules

Components of the extracellular matrix have also been implicated in maintaining the periodontal ligament as a nonmineralized fibrous connective tissue. Glycosaminoglycans (possibly dermatan sulfate) have been implicated in maintaining the periodontal ligament since enzymatic removal of these matrix components has been reported to result in deposition of calcified crystals between collagen fibrils in an *in vitro* model (Kirkham *et al.* 1995).

A collagen-associated protein (CAP) containing the cell attachment RGD sequence termed RGD-CAP has been identified in the periodontal ligament and has been proposed to play a role in preventing its mineralization (Ohno *et al.* 2002). When periodontal ligament fibroblasts were induced to express alkaline phosphatase, the expression of mRNA for RGD-CAP decreased. In addition, when RGD-CAP was added to periodontal ligament cells, their capacity to express alkaline phosphatase and form mineral nodules was suppressed. Together

these observations suggest that the presence of RGD-CAP in the periodontal ligament could prevent mineralization.

Transcription factors

Apart from soluble mediators, intracellular mechanisms involving various transcription factors that regulate osteogenic activity may be involved in preventing mineralization of the periodontal ligament. For example, expression of the homeobox protein MSX-2 by periodontal ligament fibroblasts, which is higher than in osteoblasts, may prevent the differentiation of periodontal ligament fibroblasts into osteoblasts by suppressing the transcriptional activity of Runx-2/OSF-2 (Yoshizawa *et al.* 2004). Accordingly, it has been proposed that MSX-2 may play a central role in preventing mineralization of the periodontal ligament.

Another transcription factor implicated in periodontal ligament width maintenance is a basic helix-loop-helix called TWIST. Expression of TWIST in the periodontal ligament has been noted to localize specifically along the alveolar bone interface (Komaki *et al.* 2007). Periodontal ligament cells have been shown to express the gene for TWIST, and this is higher than for osteoblast-like cells. Furthermore, when periodontal ligament cells are stimulated to express osteoblast-like genes, the expression of mRNA for TWIST decreases. This observation has been corroborated in studying siRNA to knock down TWIST gene expression in periodontal ligament cells, which demonstrated an increase in expression of mRNA for alkaline phosphatase, osteopontin, and bone sialoprotein (Komaki *et al.* 2007). While still under investigation, these effects are thought to arise from interactions between TWIST and Runx-2, which prevent DNA binding and activation of bone-protein related genes by Runx-2 (Bialek *et al.* 2004). From these observations, it was concluded that TWIST negatively influences differentiation of periodontal ligament cells into osteoblasts.

Notwithstanding all of the findings discussed in this section, the mechanism(s) controlling how the periodontal ligament remains nonmineralized, despite its interspersed between two mineralized tissues (cementum and alveolar bone), remain(s) largely speculative and unsolved.

Mechanical loading and the periodontal ligament

One of the principal functions of the periodontal ligament is to enable swift and appropriate responses to the stresses and strains placed on it as a result of natural mechanical forces generated by mastication, bruxing,

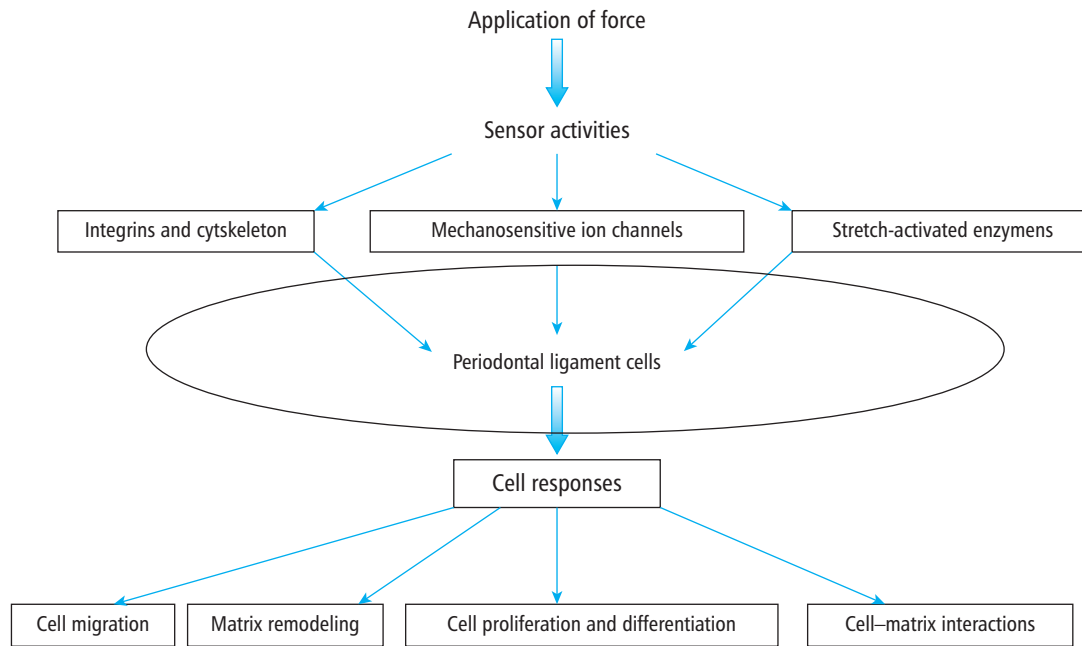


Figure 26.4 Mechanical loading and the periodontal ligament. Periodontal ligament cells perceive and respond to forces applied to the periodontium by mechanotransduction. This process involves several sensory detection mechanisms that lead to intracellular changes and subsequent responses via cell–matrix interactions, cell proliferation, extracellular matrix remodeling, and cell migration. (Adapted from Ko & McCulloch, 2001, with permission from Elsevier.)

and artificial forces such as orthodontics. These responses are largely due to the resident periodontal ligament cells that can perceive and respond to mechanical forces (Figure 26.4).

In vitro and in vivo studies have demonstrated that periodontal ligament fibroblasts can perceive and respond to mechanical stress. While considerable headway has been made in recent years in understanding the molecular events associated with the cellular responses, this is still a poorly understood area. Nonetheless, numerous histological studies of the tissue changes that occur during orthodontic tooth movement have provided a conceptual understanding for the reaction of the periodontium to mechanical loading. In essence, the periodontium responds to both pressure and tension forces, and while the molecular and cellular reactions associated with “pressure” and “tension” are now understood to be very complex, the fundamental principles remain the same. That is, on the pressure side of the periodontal ligament there is disturbance of the blood flow, cell death, and hyalinization of the connective tissue that is subsequently removed by macrophages and resorption of the alveolar bone. On the tension side there is stretching of the periodontal ligament fibers, increased blood flow, and associated deposition of bone. With these two processes acting together, forces applied to teeth result in tooth movement (either pathologic or orthodontic).

In reality, this description is very simplistic. Understanding the transmission of physical forces to tissue response is very important. This process is known as *mechanotransduction* and involves the processes that result in the biochemical reactions of a cell to physical forces (Figure 26.4). Initially, cells can respond to mechanical forces through a variety of mechanical sensors such as mechanosensitive ion channels, integrins linked to the cytoskeleton, or stretch-activated enzymes (Ko & McCulloch 2001). Through a number of downstream intracellular events, mechanical stresses within the extracellular matrix are communicated to the cell, and a feedback system involving myriad cytokines is activated to bring about tissue remodeling. Multiple cell-signaling pathways have been proposed to mediate the cell responses to mechanical stress.

Ion channels

Stretch-activated ion channels have been implicated in the response of cells to mechanical strain. These channels, which open upon pressure, allow a critical efflux of Ca^{2+} and K^{+} and prevent membrane disruption due to excessive osmotic pressure (Ko & McCulloch 2000). Stretch-activated ion channels are regulated by the intracellular actin fiber network (Pender & McCulloch 1991). Release of arachidonic acid upon application of tensile forces has also been shown to regulate the K^{+} channels in periodontal ligament fibroblasts (Saeki *et al.* 2007).

Prostaglandins

The prostaglandins were among the first of the soluble mediators proposed to play a role in mechanical force transduction (Hong & Levine 1976). It was proposed that conformation changes in the cell membrane, as a result of the application of mechanical forces, resulted in exposure of phospholipids to the action of phospholipases and release of arachidonic acid. This theory was subsequently confirmed by in vitro deformation of osteoblasts that was observed to activate phospholipase A₂, release of arachidonic acid, and subsequent PGE₂ synthesis. These events were then noted to activate cyclic AMP and an increase in intracellular Ca⁺⁺ (Harell *et al.* 1997). Prostaglandin is now a well-recognized mediator that can act in both an autocrine and paracrine manner to influence a number of cellular responses including cell division, DNA synthesis protein synthesis, and cell behavior (Yamaguchi *et al.* 1994).

Cyclic AMP

Cyclic AMP (cAMP) is another well-known mediator in mechanical force transduction. Application of stretching forces, in vitro, to periodontal ligament fibroblasts was demonstrated to result in elevated synthesis of PGE₂ and cAMP (Ngan *et al.* 1990). Since the production of cAMP was secondary and dependent upon the newly synthesized PGE₂, it was concluded that locally produced autocrine factors such as PGE₂ can modify the effect of mechanical stress on periodontal cells via the cAMP pathway.

Adenosine triphosphate

A role for adenosine triphosphate (ATP) in force transduction has been reported in more recent studies. ATP is a critical co-enzyme involved in intracellular energy transfer. It can act as an intracellular signaling molecule via its conversion to cAMP by adenylyl cyclase or, if secreted extracellularly, it can bind to P2 purinoceptors to mediate its effects. An early intracellular event following application of mechanical force to the periodontal ligament is the release of ATP (Wongkhantee *et al.* 2008). The release of ATP has been associated with expression of both osteopontin and receptor of nuclear factor- κ B (RANKL) that are associated with bone deposition and resorption, respectively (Wongkhantee *et al.* 2008; Luckprom *et al.* 2010). Both of these responses appear to be mediated through the P2Y1 purinoceptor receptor and subsequent intracellular signaling. For osteopontin, Rho kinase is activated through the binding of extracellular ATP to the P2Y1 receptor, resulting in the upregulation of osteopontin (Wongkhantee *et al.* 2008). The relationship between ATP and RANKL expression appears to be

more complex, whereby extracellular ATP acts through a process dependent on binding to the P2Y1 receptor and subsequent activation of the NF κ B-COX-RANKL axis (Luckprom *et al.* 2010).

Cell-matrix interactions

Cells interact with the extracellular matrix through a number of cell surface molecules, of which the integrins are particularly important. Integrins are composed of two structural subunits (*a* and *b*) that form heterodimers. Depending on their combination of subunits, integrins interact with RGD amino acid sequences in specific matrix components including collagens laminin, vitronectin, and fibronectin (Ruoslahti & Pierschbacher 1987). These strong interactions between cell and matrix enable cells to detect distortional changes resulting in changes in cytoskeletal arrangements, intracellular signaling mechanisms.

Cytokines

Cytokines are critical biological mediators and are responsible for a very wide range of cell activities and responses. Cytokines include the interleukins, tumor necrosis factors, interferons, growth factors, and colony-stimulating factors. They can exert their effects in both autocrine and paracrine pathways. Periodontal ligament cells have been shown to release a wide array of cytokines in response to mechanical stress and strain, but the results obtained from these studies have been confusing since the responses are many and varied. Tensile stress has been reported to both increase and decrease cytokine production by periodontal ligament fibroblasts (Saito *et al.* 1991; Long *et al.* 2001). Nonetheless, there does seem to be some differential response by periodontal ligament fibroblasts to tensile or compressive pressure. Application of continuous tensile strain results in increased synthesis of matrix metalloproteinases (MMP-1 and MMP-2) and their inhibitors (TIMP-1 and TIMP-2), decreased alkaline phosphatase activity, and increased type I collagen and fibronectin synthesis (Howard *et al.* 1998; Chiba & Mitani 2004). On the other hand, compressive forces have been reported to stimulate RANKL mRNA expression by periodontal ligament fibroblasts via increased PGE₂ synthesis and the culture supernatant contained factor(s) that stimulated osteoclastogenesis by peripheral blood mononuclear cells (Kanzaki *et al.* 2002). These findings were interpreted to indicate that periodontal ligament fibroblasts can influence osteoclast differentiation and mediate tissue changes in response to compressive forces.

More recently, rather than study individual cytokines and their release in response to mechanical forces, larger screening assays for groups of cytokines have been

carried out. In one study, the response to cyclic deformation of periodontal ligament on the expression of 70 cytokine and growth factor genes was assessed (Pinker-ton *et al.* 2008). A number of interleukins, cytokines, and growth factors were noted to be upregulated and down-regulated. However, interpretation of the results is difficult even when using this technology, and little is yet really understood. Continued study will determine whether these genes are translated to produce proteins that are not only active but also strategically located throughout the periodontal ligament consistent with the age-old observation of bone resorption on the pressure side and bone deposition on the tension side.

Conclusion

There is no doubt that the periodontal ligament is a special organ. Due to its strategic location between the tooth surface and alveolar bone, its unique cell composition and ability to repair, regenerate, and remodel itself, depending on the type of injury or forces applied to it, make it one of the most adaptive tissues in the mammalian body. There is still a great deal to be learned about this tissue and about the mechanisms of tissue homeostasis and physiology.

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Clinical correlate: two cases of destructive periodontal disease

Rahime Meral Nohutcu

Periodontal ligament in health and disease

The periodontium defines the tissues supporting and investing the tooth, including the root cementum, periodontal ligament, alveolar bone, and gingiva. The periodontium is a unique environment in the body in that two mineralized tissues in close proximity—bone and cementum—interface with a nonmineralized tissue, the periodontal ligament (PDL) (Fiorellini *et al.* 2006). Because of this unique specification, the periodontium can be considered an organ system composed of two hard tissues (cementum and bone) and two soft tissues (periodontal ligament and gingiva), which together maintain adequate function of the tooth (Bartold & Narayanan 1998; Chapter 26, this volume). The main function of the periodontium is to attach the tooth to the bone tissue of the jaws and to maintain the integrity of the surface of the masticatory mucosa of the oral cavity. Because the periodontium plays a pivotal role in the support of the tooth root within the alveolar socket, every effort must be made to maintain all of its components' vitality and function (Nanci & Bosshardt 2006).

The PDL is one of the main components of the periodontium and is continuous with the gingival connective tissue above the alveolar crest and with the pulpal tissue at the apical foramen. These continuities are important for understanding the progression of gingivitis to periodontitis and inflammation originating from pulpal tissues to the periodontal ligament and other apical supporting tissues. The periodontal ligament is a highly specialized, dense fibrous connective tissue between the cementum covering the root surface of the tooth and the alveolus and firmly binds the tooth root to the surrounding alveolar bone (Beersten *et al.* 1997; Berkovitz 2004).

The PDL not only has an important role in supporting teeth, but also contributes to tooth nutrition, homeostasis, and repair of damaged tissues. It is a cell reservoir for tissue homeostasis, repair, and regeneration. PDL has several major functions (Berkovitz 2004):

- PDL provides attachment between the tooth and alveolar bone. Having this function, it is responsible for resisting displacing forces and protecting the dental tissues from damage resulting from excessive occlusal forces.
- PDL plays role in the mechanisms of tooth eruption and drift. Thus, the tooth attains and then maintains its functional position.
- Cells within the PDL maintain the PDL region and contribute to repair of alveolar bone and cementum.
- PDL's mechanoreceptors are involved in the neurological control of mastication.

Similar to other connective tissues, PDL consists of cells and extracellular compartment comprising collagenous and noncollagenous matrix components (see Chapter 26, this volume).

With the development of inflammatory periodontal diseases, many pathological qualitative and quantitative changes occur in the molecular composition of periodontal tissues, including the PDL (Kirkham *et al.* 1991, 1995). Inflammatory periodontal diseases cause marked destruction of collagens in the extracellular matrix of PDL. With the developing inflammatory lesion, the gingival collagen becomes more soluble. In chronic periodontal diseases, the concentration of collagen has been shown to be decreased dramatically when compared to healthy periodontium. With the progression of

periodontal disease, the nature and the ratio of collagen types begin to change, with the amount of type V collagen increasing. A new collagen, type I trimmer, may also appear (Berkovitz 1990, 2004; Bartold & Narayanan 2006). The collagen fibril diameter has also been reported to change with the developing inflammatory lesion (Kirkham & Robison 1995).

Following the onset of periodontal disease, there may be a change in the content of ground substance, with chondroitin sulphate content increasing and dermatan sulphate decreasing (Berkovitz 2004). Quantitative and qualitative changes also occur to the gingival proteoglycans, but these are not as marked as those noted for the collagens. During the development of periodontitis, there is evidence of degradation of both proteoglycan core proteins and hyaluronic acid and a significant change in the types of proteoglycans within inflamed periodontal tissues. For example, significant changes in the distribution of decorin and syndecans in inflamed human gingiva have been reported (Berkovitz 2004; Nanci & Bosshardt 2006).

Left untreated, the host-immune response results in severe destruction of the PDL and loss of supporting

bone and eventually exfoliation of teeth. Described in this chapter are two types of periodontal disease associated with inflammation of periodontal tissues and bone loss.

Case 1: chronic periodontitis

Periodontitis is one of the most common inflammatory diseases of humans and of tooth loss in adults. It is characterized by progressive destruction of the tooth-supporting apparatus including the gingiva, alveolar bone, cementum, and periodontal ligament. If left untreated, it can lead to loosening and subsequent loss of teeth (Pihlstrom *et al.* 2005; Nanci & Bosshardt 2006). Chronic periodontitis is the most prevalent form of periodontitis, generally considered to be a slowly progressing disease. In general, clinical features of untreated chronic periodontitis may include supragingival and subgingival plaque accumulation, calculus formation, gingival inflammation, periodontal pocket formation, loss of periodontal attachment, and alveolar bone loss (Figure 27.1). In this chronic periodontitis case of a 40-year-old Caucasian male, with poor oral hygiene, the gingiva is



Figure 27.1 Clinical view of generalized chronic periodontitis in a 40-year-old male with a 10-year history of smoking at least one pack of cigarettes per day. All teeth have a considerable amount of plaque and calculus. A. Frontal view. B–C. Buccal view of the left and right regions, respectively. D–E. Palatal (maxillary) and lingual (mandible) view, respectively.

moderately swollen and exhibits changes in surface characteristics including loss of stippling and rolled gingival margins. Cratered papillae and gingival recession are marked as a result of attachment loss and alveolar bone destruction. Clinical examination revealed periodontal pockets, with generalized attachment loss, probing depths greater than 5 mm especially in the molar regions, mobility, and bleeding and suppuration from the pockets. The reason for tooth mobility is most likely due to destruction of the PDL combined with loss of supporting alveolar bone.

Generalized chronic periodontitis is characterized by widespread periodontal damage, usually in older individuals, and a slow rate of disease progression is assumed based on the relatively low ratio of damage to age (Novak & Novak 2006). The disease can be diagnosed radiographically by evidence of bone loss, and periodontal ligament loss undoubtedly accompanies bone loss (Figure 27.2). Radiographs showed a generalized and horizontal pattern of bone loss, which is characteristic of generalized chronic periodontitis.

Case 2: aggressive periodontitis

Aggressive periodontitis causes rapid destruction of the periodontal attachment apparatus including periodontal ligament fibers and the supporting alveolar bone. Aggressive periodontitis may be localized or generalized, and it is often seen in younger individuals (Ranney 1993). The localized form of the disease was reported by Gottlieb as “diffuse atrophy of the alveolar bone” in 1923. The disease was characterized by loss of collagen fibers in the periodontal ligament and their replacement by loose

connective tissue and extensive bone resorption, resulting in a widening periodontal space (Novak & Novak 2006). Generalized form of the disease is characterized by a more widespread pattern of destruction. The rapid progression of attachment and bone loss, which differentiates it from chronic periodontitis, is the primary characteristic of aggressive periodontitis (Ranney 1993).

A remarkable characteristic of aggressive periodontitis is the lack of marked clinical inflammation despite the presence of deep periodontal pockets and advanced bone loss. In many aggressive periodontitis cases, the amount of plaque on the affected teeth is minimal, and it seems inconsistent with the amount of periodontal destruction present (Lang *et al.* 1999; Novak & Novak 2006). Generalized aggressive periodontitis in a 33-year-old, otherwise healthy, Caucasian male patient with a family history of early tooth loss due to periodontal disease, showed severe, generalized bone loss despite the limited plaque accumulation and limited inflammation of the gingival tissues (Figures 27.3 and 27.4). Radiographs present severe and generalized bone loss including vertical and angular components. Inconsistencies between the clinical amount of deposits on teeth and the severity of periodontal destruction are obvious. The rate of bone loss in aggressive periodontitis is about 3–4 times faster than in chronic periodontitis. Due to the rapid destruction of attachment apparatus and supporting tissues, the overall prognosis for these patients is poorer than that for patients with chronic periodontitis.

Chronic and aggressive forms of periodontitis both involve progressive destruction of the periodontium; left untreated, the destruction of periodontal ligament and



Figure 27.2 Radiograph of the chronic periodontitis case seen in Figure 27.1. Radiographs show a generalized, horizontal pattern of bone loss.



Figure 27.3 Generalized aggressive periodontitis in 33-year-old otherwise healthy male prior to therapy. Intraoral photographs show minimal plaque accumulation and limited inflammation of the gingival tissues.



Figure 27.4 Radiograph of the aggressive periodontitis case seen in Figure 27.3 showing severe, generalized bone loss. Some of the teeth have already been lost due to the advanced disease.

alveolar bone may result in loss of the affected teeth. It is currently believed that most of the destruction found in all cases of periodontitis is mediated by host inflammatory and immunological responses to subgingival biofilms (Armitage *et al.* 2010). Both diseases share some major characteristics:

- Acute inflammatory changes in response to microbiological colonization of the tooth
- Influx of neutrophils toward microbial components of subgingival biofilms
- Detachment of junctional epithelium and its conversion to pocket epithelium
- Inflammatory destruction of connective tissue adjacent to the pocket epithelium
- Accumulation of chronic inflammatory cells, and apical migration of the epithelium onto the tooth root

- Osteoclastic resorption of alveolar bone (Novak & Novak 2006)

Although similar in many general or overall respects, it has been suggested that chronic and aggressive forms of periodontitis have a number of significant clinical differences including age of onset, rates of progression, patterns of destruction, clinical signs of inflammation, and relative abundance of plaque and calculus.

Tissues obtained from chronic and aggressive forms of periodontitis appear identical when viewed using conventional light and electron microscopic methods. It has been suggested that PDL collagen loss noted in chronic periodontitis can be a consequence of two factors: disorganized fiber bundles and reduced ability of ligament fibroblasts to form collagen precursors (Smith *et al.* 2010).

Future considerations for regenerating tissues

It has long been suggested that the PDL is of central importance in determining the outcome of periodontal regenerative procedures because of the fact that the PDL provides important functions during periodontal wound healing. Regeneration of periodontal tissues involves three diverse and unique tissues: the periodontal ligament, cementum, and alveolar bone (Polimeni *et al.* 2006). During periodontal regeneration, the remaining healthy periodontal ligament plays a key role in the regeneration of these new compartments. The regeneration capacity of the PDL itself is attributed to a few progenitor cells maintaining their proliferation and differentiation potential in the periodontium (Bartold *et al.* 2000). In fact, PDL tissues remaining on the root surface after a tooth extraction contain undifferentiated cells that have the ability to regenerate alveolar bone and PDL and thus can be a cell source for alveolar bone tissue engineering (Seo *et al.* 2004; Gronthos *et al.* 2006; Hosoya *et al.* 2010; Ikeda *et al.* 2011). Furthermore, PDL provides a model system to study connective tissue homeostasis and remodeling because of the rapid remodeling of extracellular matrix proteins in the PDL. In the future, improvements in the ability to diagnosis disease susceptibility and early progression coupled with improvements in therapeutic modalities should enable us to decrease periodontal disease within our communities.

Summary

Periodontitis is one of the most prevalent infectious diseases characterized by progressive destruction of the tissues that support the teeth such as alveolar bone, cementum, and PDL. The interaction between these components not only determines tissue health, but also

reflects events associated with tissue damage, repair, and regeneration. The PDL as a highly specialized connective tissue is considered to be one of the main components of the periodontium. PDL not only has a pivotal role in supporting teeth but also plays a role in tooth nutrition, homeostasis, and regeneration and repair of damaged periodontal tissues. Major structural and compositional changes occur in the PDL with the development of inflammatory periodontal diseases. If periodontal diseases are left untreated, with the concomitant destruction of PDL, attachment apparatus, and alveolar bone, exfoliation of teeth is inevitable.

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Periodontal disease and inflammation-induced bone remodeling

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Bacterial etiology of periodontal disease

Periodontal diseases include gingivitis and periodontitis. Both are triggered by bacteria and both involve a host response. However, in gingivitis the destruction of tissue caused by the inflammatory response to bacteria is reversible and does not involve bone loss, while in periodontal disease, there is irreversible loss of connective tissue attachment to the root surface and bone loss. Pre-clinical and clinical studies clearly implicate a bacterial etiology of gingivitis and periodontal diseases involving bone loss. Although bacteria are required for induction of periodontal disease, bacterial infection per se does not induce bone loss without the host response (Graves *et al.* 2011). Studies in animals demonstrate a relationship between microbial plaque, inflammation, and periodontal bone loss (Keyes & Jordan 1964; Saxe *et al.* 1967; Lindhe *et al.* 1975). Experimental gingivitis studies provide proof that accumulation of microbial biofilm on the tooth surfaces causes the activation of the inflammatory process around gingival tissue (Loe *et al.* 1965). In these investigations, inflammation continues as long as the microbial biofilm is present adjacent to the gingival tissues; clinical (but not histologic) signs of inflammation resolve following removal of the accumulated plaque.

Animal studies have confirmed the bacterial etiology of periodontal disease and have provided further evidence that inflammation is critical in the pathologic process. In a ligature-induced experimental model of periodontitis, the placement of a ligature around the

teeth causes plaque accumulation and facilitates bacterial penetration that leads to gingival inflammation (gingivitis) and alveolar bone resorption (periodontitis) (Graves *et al.* 2008). In contrast, the placement of ligatures in gnotobiotic rats does not cause significant increases in gingival inflammation or periodontal bone loss (Rovin *et al.* 1966), establishing the essential role of bacteria in initiating the process. Furthermore, treatment of animals with antibiotics or topical application of chlorhexidine reduces the bacterial load and significantly reduces bone resorption in this model (Weiner *et al.* 1979; Kenworthy & Baverel 1981); increasing the bacterial load enhances periodontal breakdown (Nagahata *et al.* 1982). In other animal models, the inoculation of periodontal pathogen *Porphyromonas gingivalis* into the oral cavity induces alveolar bone resorption in the mouse (Baker *et al.* 1994, 1999, 2000; Lalla *et al.* 1998). Similarly, inoculation of the oral cavity of mice with *Aggregatibacter actinomycetemcomitans* (Garlet *et al.* 2006) or *Tannerella forsythia* (Sharma *et al.* 2005) stimulates periodontal bone loss. Introduction of *A. actinomycetemcomitans* in rats leads to colonization and the loss of alveolar bone (Schreiner *et al.* 2003; Fine *et al.* 2005). Thus, experimental studies in animal models support the human clinical trials implicating bacteria in the initiation of inflammation and periodontal disease (Reddy *et al.* 2003; Kirkwood *et al.* 2007).

Pathologic changes in the periodontium are associated with the presence of specific bacteria on the tooth surface, in the sulcus, or within the tissues. These organisms are capable of synthesizing products (e.g.,

collagenase and other proteases, hyaluronidase, and chondroitin sulfatase) that cause damage to epithelial and connective tissue cells, as well as to intercellular constituents, such as collagen, ground substance, and glycocalyx (cell coat). Their production is thought to facilitate invasion of connective tissue through junctional epithelial cells during early gingivitis. Following invasion, the host response to bacteria stimulates degradation of the connective tissue (Caffesse & Nasjleti 1976). The essential role of the host response in periodontal breakdown consisting of loss of connective tissue attachment to the root surface and bone loss has been demonstrated by studies in which the host response is inhibited (Graves 2008). This reduction may occur even if antibacterial defenses are weakened.

Activation of the innate immune response and periodontal disease

In the periodontium, both the innate and adaptive responses are triggered by bacteria. A consequence of the constant attempts to eradicate bacteria is periodontal tissue destruction. The progression from gingivitis to periodontitis is likely due to a combination of several factors, including the persistence of the causative organisms and high levels of pro-inflammatory mediators produced by tissue infiltrating inflammatory cells (Graves *et al.* 2011). This process involves the recruitment and activation of leukocytes of the innate immune response (neutrophils, macrophages, dendritic cells, etc.) and the adaptive immune response (T-cells, B-cells, or plasma cells; Figure 28.1). Although the immune response leads to periodontal disease, ultimately it is needed to protect the host from invasion by oral bacteria that could be potentially lethal if they were not held at bay.

The innate immune response occurs through activation of pattern recognition receptors, for example Toll-like receptors (TLR) that broadly recognize microbes in a nonspecific manner. The innate immune response is immediate and present in all animals. The innate immune response includes several cell types and the action of inflammatory mediators such as cytokines and prostaglandins (PGs) and antibacterial factors such as complement and defensins (Figure 28.1). In addition to providing protection from microbial infection, the innate immune cells remove foreign substances, damaged tissue, and dead cells. They also set the early stages of wound healing. Cells of the innate immune response produce mediators such as prostaglandin E_2 (PGE₂); cytokines such as interleukin-6, tumor necrosis factor (TNF), and interleukin-1 (IL-1); and a number of different chemokines that induce recruitment of leuko-

cytes. These factors have been implicated in inflammation, loss of connective tissue attachment, and periodontal bone resorption (Graves *et al.* 2011).

There is compelling evidence that PGs and other metabolites of arachidonic acid contribute to periodontal disease. Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the production of inflammatory mediators primarily by blocking the enzyme systems that convert arachidonic acid to the individual metabolites. Lipid mediators most commonly associated with inflammatory bone resorption are PGs and leukotrienes, which are synthesized via processing of membrane-anchored lipids by genome-encoded enzymes such as 5-lipoxygenase (5-LO) and cyclooxygenase (COX). Leukotriene B₄ (LTB₄) can induce osteoclast differentiation of precursor cells independently of RANKL. PGE₂ has been associated with inflammatory bone resorption (Miyachi *et al.* 1992), but it may also have bone-sparing effects through direct activity on osteoclasts. PGE₂ and LTB₄ levels are elevated in periodontal diseases, and the increase in their levels was shown to accompany the development of experimental gingival inflammation in humans. Systemic application of many NSAIDs, including flurbiprofen, ibuprofen, indomethacin, and naproxen, decreased periodontal bone loss (Williams *et al.* 1985; Jeffcoat *et al.* 1988). Although relatively low-dose systemic NSAID therapy has been successfully used to slow the rate of alveolar bone loss in periodontitis, its use is not without side effects, predominantly gastrointestinal irritation (Paquette *et al.* 2000). The role of PGE₂ in bone resorption is further demonstrated by the fact that mice lacking PGE₂ receptors or PGE₂-synthase present reduced inflammation- and LPS-induced bone resorption (Suzawa *et al.* 2000). More recent evidence indicates that other arachidonic acid products, lipoxins and resolvins, reduce inflammation and alveolar bone loss in experimental periodontitis (Hasturk *et al.* 2006). Collectively, the modulation of the host response indicates that it has an essential role in bacteria-induced periodontal bone resorption.

Cytokines play an important role in bone resorption mediated by periodontal bacteria. In human longitudinal studies, IL-1 and IL-6 are elevated in gingival crevicular fluid (GCF) at sites of recent bone and attachment loss (Lee *et al.* 1995). Pro-inflammatory cytokines IL-1 β and TNF α contribute to loss of connective tissue attachment and bone resorption as shown in a ligature animal model in nonhuman primates (Assuma *et al.* 1998; Delima *et al.* 2001). TNF and IL-1 specific antagonists reduce the inflammatory cell infiltrate that forms in close proximity to bone, bone resorption, and connective tissue attachment in this model. Moreover, inhibition of IL-1 alone significantly reduces the migration of

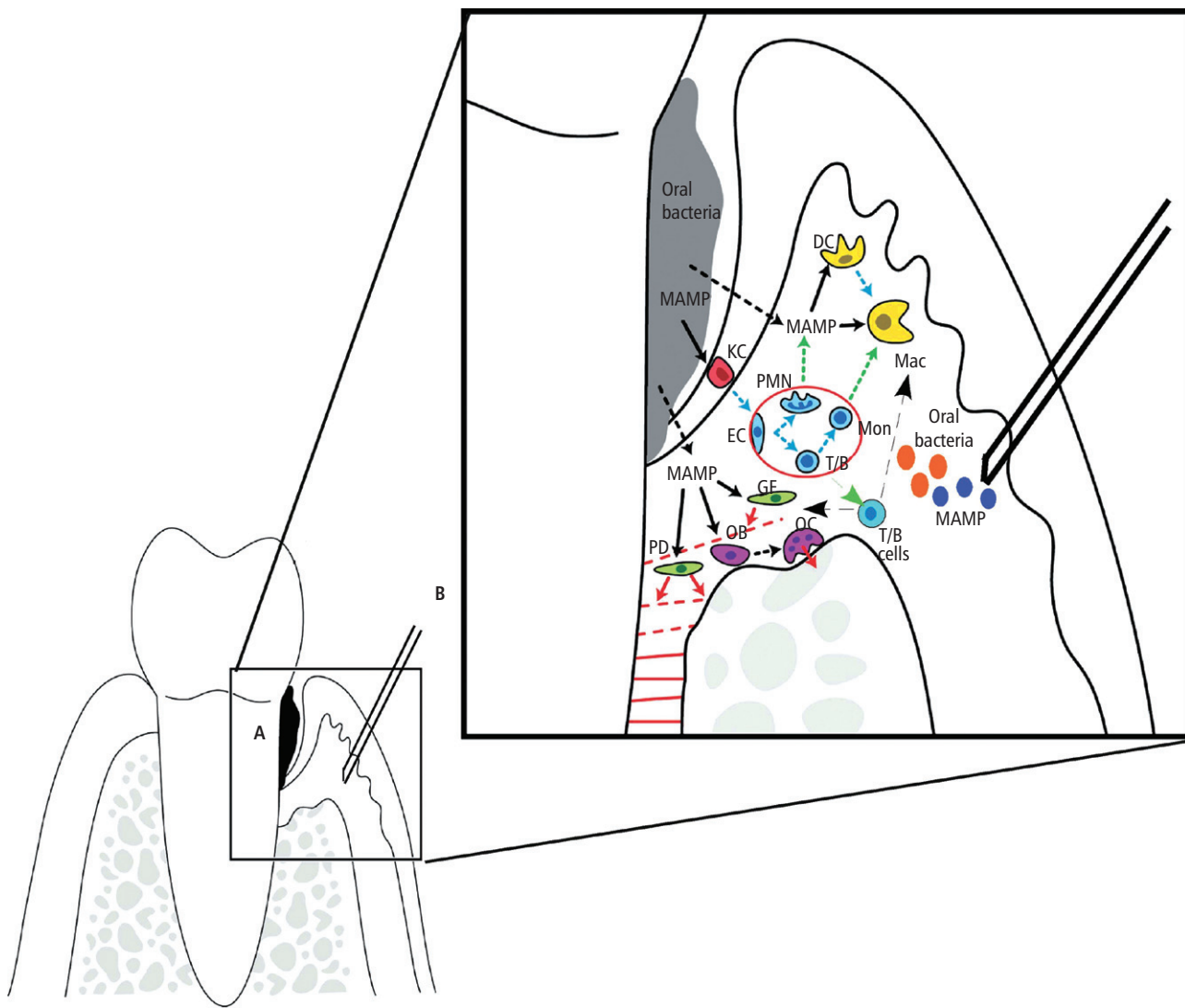


Figure 28.1 Antigen presentation to naïve CD4 cells leads to development of committed CD4 cells. Cells of the innate immune system (antigen-presenting cells, monocytes and macrophages, neutrophils, etc.), CD4 cells, and, to a lesser extent, B-cells produce differing cytokine patterns that can influence each other's level of activation. Dependent on the predominant cell type and cytokine milieu, these cells may have either stimulatory or inhibitory influences on the maturation of osteoclast precursor cells, osteoclast activity, and resultant bone resorption.

an inflammatory infiltrate toward bone and the loss of alveolar bone (Delima *et al.* 2002). In another model, TNF receptor-deficient mice have significantly reduced periodontal loss and *P. gingivalis*-induced osteoclastogenesis compared to wild-type mice (Graves *et al.* 2001; Garlet *et al.* 2007). Similarly, IL-6 deficient mice have reduced alveolar bone loss in a *P. gingivalis* oral infection model (Baker *et al.* 1999). Overexpression of IL-1 in gingival epithelium promotes periodontal bone loss (Dayan *et al.* 2004). Likewise, application of TNF increases periodontal bone loss in a rat ligature model (Gaspersic *et al.* 2003). Thus, mediators associated with the innate immune response including pro-inflammatory arachi-

donic acid metabolites and cytokines (TNF, IL-1, and IL-6) are induced during experimental periodontitis (Koide *et al.* 1995) and have been shown in gain of function and loss of function experiments to contribute to periodontal tissue destruction.

Activation of the adaptive immune response and periodontal disease

As opposed to the cells and mediators of the innate immune system, the adaptive immune response has a level of sophistication that allows for a high degree of specificity and modification of its activity based on prior

exposure. This allows for immunologic memory and the ability to distinguish self from nonself. The adaptive immune response involves a number of different cells and receptor molecules. B- and T-lymphocytes, derived from multipotent hematopoietic stem cells, play a large role in the humoral and cell-mediated immune responses, respectively. The naïve B- and T-cells leave their respective sites of origin in the bone marrow or thymus and enter the lymphatic system. Lymphocytes are presented antigen by antigen-presenting cells (APCs; dendritic cells, mature B-cells, and to a lesser extent macrophages). These “professional” APCs are able to modulate the responding lymphocytes by expressing auxiliary co-stimulatory molecules that interact with receptors on lymphocytes to regulate the intensity and the duration of their responsiveness to a particular antigen. Through central and peripheral tolerance, autoimmunity is prevented.

Several lymphocyte subsets have been identified that have differing functions. The type of lymphocyte response is dictated by the context in which the APC first encounters the antigen. T-cells are generally stimulated to become either “cytotoxic” CD8+ cells or “helper” CD4+ cells. Tissue-based dendritic cells or macrophages will locally engulf exogenous pathogens such as bacteria or parasites and then migrate, via chemotactic signals, to regional lymph nodes. Blood-borne pathogens are similarly handled by resident APCs in the spleen. The APC will then digest the macromolecular constituents of these pathogens into small molecular sequences that can be displayed on their surface by attaching them to a “self”-receptor, the major histocompatibility complex (MHC), known in humans as the human leukocyte antigen (HLA). The specificity of these cells is insured by the engagement of a unique T-cell receptor (TCR), which will interact with the specific peptide antigen expressed in conjunction with the MHC molecule. Once appropriately activated, lymphocytes will multiply and mature into effector cells. After the infection has been controlled, the major expansion of the specific T-cells will regress, but a small component of this population of cells will be retained as memory cells capable of mounting a more rapid and aggressive response should there be a second encounter with the same antigen (Figure 28.2).

The CD4+ lymphocytes, or helper T-cells, that develop as a consequence of exposure to exogenous antigens are capable of curtailing infections through different mechanisms. The helper T-cells express TCR that will recognize antigen bound to Class II MHC molecules. Depending on the type of APC, the nature of the co-stimulation, and the cytokine milieu in which the T-cell is activated, the CD4+ cell will mature into differ-

ing subsets that are capable of directing immune responses to different types of pathogens in part through the production of unique cytokine profiles. The three major types of effector CD4+ T-helper cell responses are designated Th1, Th2, and Th17, each particularly effective in controlling the elimination of different types of pathogens (intracellular pathogens, parasites, and extracellular pathogens, respectively). The Th1 response is characterized by the production of interferon- γ and TNF α , which augment cytotoxic T-cell responses, opsonize antibody production, and activate macrophage responses. Th2 responses are characterized by the production of IL-4, which activates antiparasitic B-cell responses, IgE production, and eosinophilic infiltration. The more recently recognized Th17 response, which requires the presence of transforming growth factor beta (TGF β), IL-6, and IL-23, results in production of IL-17 and subsequent stimulation of neutrophil function to evoke a highly pro-inflammatory response to extracellular pathogens. In addition, a fourth type of CD4+ cell, regulatory T-cells (T-regs), has been identified. In contrast to the other types of effector CD4+ cells, these cells serve to modulate activation, proliferation, and effector function of conventional T-cells possibly mediated through production of the anti-inflammatory cytokines IL-10 and TGF β , and thus seem to control responses to self-antigen, which can result in the development of autoimmune diseases. (Vignali *et al.* 2008) There are additional T-cell populations that defy easy categorization, including $\gamma\delta$ T-cells that possess an alternative TCR, as opposed to CD4+ and CD8+ $\alpha\beta$ T-cells, and natural killer (NK) cells that share characteristics with cytotoxic T-cells; they do not employ the typical antigen recognition pathways but do have features of both the innate and adaptive immune responses.

It has become clear that CD4+ T-cells are critically involved in establishing an effective defense against invading periodontal pathogens and that periodontal pathogens, to varying extents, have been demonstrated to stimulate not only Th1 and Th2 cells, but also the newly described Th17 and T-regs (Teng *et al.* 2000; Kawai *et al.* 2006; Rauner *et al.* 2007; Graves *et al.* 2008). These cells are simultaneously present in infected periodontal tissues, and the cytokines locally produced contribute to the development of alveolar bone destruction (Kawai *et al.* 2006). Severe combined immunodeficient (SCID) mice, which lack both B- and T-lymphocytes, demonstrate considerably less bone resorption than wild-type mice when challenged with *P. gingivalis* (Baker *et al.* 1994). However, when mice are engrafted with human CD4 (+) T-cells from individuals with aggressive early onset periodontal disease and then challenged with

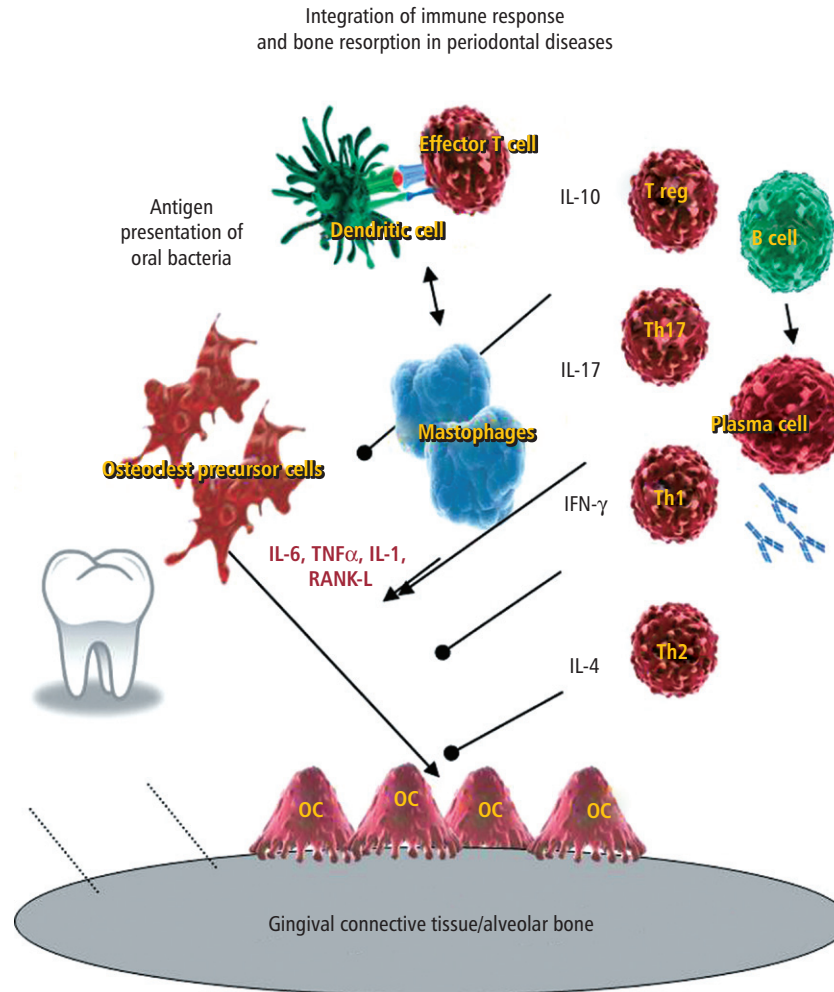


Figure 28.2 Microbe-associated molecular patterns stimulate the innate immune response. Bacteria or their products cross the epithelial barrier and penetrate into connective tissue. Bacterial components, microbe-associated molecular patterns (MAMPs), stimulate signaling by interacting with Toll-like receptors on host cells to stimulate recruitment and activation of innate immune cells such as monocytes and macrophages, dendritic cells (DCs), polymorphonuclear leukocytes (PMNs), and so on. In turn, adaptive immune response cells are recruited and activated through antigen-specific induction (T-cells and B-cells). The innate and adaptive immune response cells act cooperatively to maximize the antibacterial defense and in the process produce mediators that stimulate osteoclast (OC) formation.

A. actinomycetemcomitans, more severe periodontal bone loss occurs (Teng *et al.* 2000). In similar studies using mice with more specific immune deficiencies (CD4⁺ T-cell deficient, CD8⁺ T-cell deficient, and natural killer T-cell deficient) challenged with *P. gingivalis*, an attempt was made to further identify the specific T-lymphocyte subtypes involved. Only MHC class II-responsive CD4⁺ T-cell deficient mice had diminished bone loss compared to normal controls (Baker *et al.* 1999). Thus, in gain of function experiments (Teng *et al.* 2000) and loss of function experiments (Baker *et al.* 1999), CD4⁺ T-cells have been shown to play a role in periodontal disease progression. Moreover, an important component is the production of RANKL by these cells (Teng *et al.* 2000).

Th-17 cells appear to have contradictory roles in the development of periodontal-related bone loss. Production of IL-17 may have an important protective function since genetic deletion of IL-17 receptors in an animal model of periodontal disease leads to enhanced periodontal bone loss (Yu *et al.* 2007). In contrast, humans with periodontal disease have increased levels of Th17 cells and IL-17 mRNA, suggesting that IL-17 contributes to the destructive process (Cardoso *et al.* 2009). More recently, T-regs have been identified in periodontal tissues (Nakajima *et al.* 2005; Ernst *et al.* 2007; Cardoso *et al.* 2008), and they may reduce periodontal disease progression (Cardoso *et al.* 2008).

The involvement of T-cells is likely mediated in great part through their production of pro-inflammatory

cytokines that are capable of modulating osteoblast activity, leading to local bone resorption. In experimental models, deletion of IFN- γ in mouse models corresponds with less bone loss as compared to wild-type mice (Baker *et al.* 1999). An important lymphocyte product that mediates periodontal bone loss is RANKL. RANKL stimulates osteoclast formation, maturation, and activation as well as bone resorption and has been established as playing a pivotal role in periodontal disease (Teng *et al.* 2000; Han *et al.* 2006; Jin *et al.* 2007; Chapter 3, this volume). Under normal circumstances, local production of RANKL by osteoblasts is responsive to physiologic effectors such as hormonal activity (estrogen, calcitonin, and parathyroid hormone) and mechanical stimulation. However, under pathological circumstances such as periodontitis, RANKL is also produced by activated B-cells and T-cells (Kawai *et al.* 2006).

Counteracting the effects of RANKL is osteoprotegerin (OPG), the naturally occurring decoy molecule for RANK, also produced by osteoblasts in an attempt to fine-tune the level of osteoclastogenesis and bone resorption. The RANKL:OPG ratio has been examined in both gingival tissues and GCF in various periodontal conditions, with chronic periodontitis lesions demonstrating an increase in RANKL production and an increase in the RANKL:OPG equilibrium in favor of RANKL and consequent bone resorption (Crotti *et al.* 2003). The pivotal role of RANKL has been documented by studies in which RANKL is inhibited by using denosumab, a monoclonal antibody with specificity for RANKL, resulting in decreased alveolar bone loss (Teng *et al.* 2000; Han *et al.* 2006; Jin *et al.* 2007).

The other major lymphocyte population, B-cells, has the primary function of producing immunoglobulins, or antibodies, with specificity for epitopes, or antigen recognition sites, on particular macromolecules or pathogens. Antibodies circulate in blood or lymph, or can be secreted by B-cells resident on mucosal surfaces, and function to protect the host by neutralizing foreign antigens such as toxins, bacteria, and unincorporated viruses. Activated B-cells further differentiate into plasma cells capable of producing large amounts of specific antibody. Some B-cells are capable of responding to antigen without the necessity of T-cell assistance, but, more typically, B-cells respond to antigen in conjunction with co-stimulatory activation by helper T-cells. Whereas plasma cells are relatively short-lived cells, surviving a matter of days, memory B-cells develop and can survive for decades, retaining specificity for previously encountered antigen. Beyond the role of B-cells in the production of antibody and their ability to serve as antigen-presenting cells, as noted in this chapter, B-cells have also been

shown to have the capability to produce immunoregulatory cytokines.

In an animal model of periodontal disease, B-cells were documented to contribute to periodontal bone loss in the absence of T-cells through mechanisms mediated through the production of RANKL (Han *et al.* 2006). Adoptive transfer of B-cells from congenitally athymic rats immunized against *A. actinomycetemcomitans*, followed by an intragingival injection of the bacteria, stimulated greater alveolar bone resorption than control mice that had received B-cells from non-immunized mice. This effect was counteracted by the administration of OPG (Han *et al.* 2006). In a study of human subjects with varying degrees of periodontal disease, the level of microbial-specific human IgG response in GCF was significantly correlated with the degree of clinical attachment and bone loss in subjects with aggressive periodontitis (Liu *et al.* 2010).

Interactions between the innate and adaptive immune responses

The interactions between the innate and adaptive portions of the immune system are constant and critical, and the distinctions between them are truly blurred (e.g., B1 cells, $\gamma\delta$ T-cells). Without the benefit of the innate system, the adaptive component would function in a very indiscriminate manner or not at all. Without APCs to activate and co-stimulate T-cells and without the appropriate cytokine milieu, T-helper and cytotoxic responses would be non-existent and B-cell responses would be superficial and unfocused. Without the ability of the adaptive immune portion to quickly augment its response to novel antigens and retain memory to recall antigens, the innate system would be overwhelmed by having to respond to constant exposure with both new and old pathogens without a more sophisticated and specific immune response.

The communication between the innate and adaptive immune systems involves cell-to-cell interactions (antigen presentation) and production of soluble mediators that modulate inflammation (cytokines or chemokines). As the innate immune system is generally thought to yield more prompt but less focused immune responses, it is commonly thought that to hold the reins on the adaptive system (i.e., activation of APCs in response to microbial stimuli through TLRs or scavenger receptors) results in the production of type I interferons and the upregulation of co-stimulatory molecules that facilitate T-cell responses.

In periodontal infections, interactions between the innate and adaptive immune responses allow for a more

vigorous response to microbes. This was recently documented in a model of periodontal bone resorption involving injection of *P. gingivalis* adjacent to calvarial bone, whereby mice that had received prior immunization against the periodontal pathogen demonstrated an increased innate immune response. The addition of the adaptive immune response to the innate immune response had the consequence of increasing expression of innate immune cytokines, enhancing bone resorption, and decreasing coupled new bone formation (Behl *et al.* 2008). Even more recently, the roles of both CD4+ T-cells and B-cells were elaborated in an animal model of periodontitis involving inoculation of susceptible rats with *A. actinomycetemcomitans*. In addition to evident bone resorption, CD4+ T-cells and B-cells were increased and activated, with resultant increases in RNA expression of a broad range of cytokines implicated in bone resorption (Li *et al.* 2010).

Inflammation-induced osteoclastogenesis

In the periodontal microenvironment, a crucial step in the pathogenesis of periodontitis is the differentiation and activation of osteoclasts, a hallmark distinction between destructive periodontitis and gingivitis. The inflamed periodontal microenvironment presents all cell types necessary for osteoclastogenesis. Inflammation-induced osteoclastogenesis is a complex process because it involves multiple cell types and biological mediators. The strategies for its modulation can target primarily the stromal-immune cells for an indirect effect, or directly affect osteoclast precursor cells and osteoclasts. In the following sections, the information on different aspects of inflammation-induced osteoclastogenesis that may be therapeutically targeted will be discussed briefly. Other mechanisms relevant for inflammation-induced osteoclastogenesis are still being unraveled, such as posttranscriptional silencing mechanisms mediated by micro-RNAs, for example miR-223 (Sugatani & Hruska 2009).

As discussed above, factors that stimulate bone resorption include the RANK–RANKL–OPG axis, IL-1 β , TNF α , IL-6, arachidonic acid metabolites, and chemokines. Several inhibitors of these specific cytokines have been approved for use in humans for the treatment of rheumatoid arthritis; the effects have included inhibition of bone loss including tocilizumab (a humanized IL-6 receptor antibody), etanercept (a fusion protein of IgG with a soluble receptor for TNF α), and anakinra (a human recombinant form of IL-1 receptor antagonist) (Nishimoto & Kishimoto 2008; Mertens & Singh 2009; Wiens *et al.* 2009).

The FDA has recently approved denosumab, a recombinant human antibody against RANKL, for treatment of bone resorption associated with osteoporosis and certain metastatic cancers. In periodontal disease models, there is a relative paucity of information on in vivo modulation of osteoclastogenesis and bone resorption. Many drugs that reduce bone resorption in experimental periodontitis have drawbacks such as inhibiting the host defense, thereby enhancing the risk of infection and side effects that have untoward consequences such as gastric problems with NSAIDs or joint stiffness in rheumatoid arthritis patients caused by MMP inhibitors (Krzieski *et al.* 2007). Thus, in some cases, inhibition of select target cytokines may reduce osteoclastogenesis and bone resorption but with limited practical usefulness due to side effects.

Several intracellular signaling molecules have been targeted in reducing inflammation stimulated bone resorption. The MAP kinase pathway is important in mediating cytokine-induced osteoclastogenesis and has been shown to reduce bone loss in periodontal disease (Kirkwood & Rossa 2009). Endogenous signaling regulatory mechanisms represented by the suppressor of cytokine signaling (SOCS) proteins have been recently implicated in inflammation-induced osteoclastogenesis examining mice with genetic deletion of SOCS genes including periodontal bone loss (Menezes *et al.* 2008; Zhang *et al.* 2009). The protein kinase C (PKC) pathway has been shown to mediate osteoclastogenesis and could potentially be involved in periodontal bone loss (Lee *et al.* 2003). Similarly, a downstream target of the PKC pathway, Akt, may be important for osteoclast survival and activation (Nakamura *et al.* 1997) and could be potentially studied for its impact in periodontal disease.

Transcription factors represent other potential targets that have not been fully investigated in modulating periodontal disease. NF- κ B is a transcription factor involved in several inflammatory pathways, osteoclastogenesis, and bone resorption. LPS-induced activation of NF- κ B in pre-osteoclast cells is reported to support osteoclastogenesis (Suda *et al.* 2002), and the critical role of NF- κ B in osteoclastogenesis is demonstrated by the lack of osteoclastogenesis in mice lacking NF- κ B subunits (Iotsova *et al.* 1997). The perspective of a pharmacologic approach to inhibit NF- κ B is shown in animal models of rheumatoid arthritis with the use of a synthetic peptide disrupting the IKK complex, which prevented NF- κ B activation and significantly reduced osteoclastogenesis (Kim *et al.* 2009). Natural products that modulate signaling pathways such as curcumin and resveratrol may be useful in inhibiting osteoclastogenesis and reducing bone resorption as suggested by their potent

inhibitory effect on NF- κ B activation and also on osteoclastogenesis in vitro. In vivo studies with these natural products are relatively scarce, but they indicate a potent anti-inflammatory effect (Guimaraes *et al.* 2010, 2011) and also show an effect on bone turnover in a diabetes model (Hie *et al.* 2009; von Metzler *et al.* 2009; He *et al.* 2010).

Coupled bone formation in periodontal disease

Bone resorption in a healthy adult is followed by the formation of new bone. This process is called *coupling* and is essential for maintaining bone mass and a healthy skeleton (Parfitt 1982). For coupling to occur, it is essential to have an adequate pool of osteoblast precursors that are stimulated to expand and differentiate into osteoblasts that produce matrix. Thus, it would be expected that an episode of alveolar bone resorption would be followed by bone formation to repair the missing bone. However, this does not occur in periodontitis, where it is clear that there is inadequate coupling leading to a net loss of bone. Thus, a pathologic component of periodontitis is the failure to form new bone to replace that which is resorbed (incomplete coupling) (Figure 28.3).

Although there may be multiple mechanisms for uncoupling in periodontitis, the most obvious culprit is the inflammatory process. It is our hypothesis that inflammation present during the coupling process suppresses new bone formation and thereby causes uncoupling. Although there is no direct evidence for this in human studies, inflammatory cytokines have been shown to prevent bone nodule formation in vitro (Stashenko *et al.* 1987), and animal studies have provided consistent evidence that inflammation negatively affects bone formation in a number of pathologic conditions. For example, upregulation of the adaptive immune response enhances and increases the period of inflammation stimulated by *P. gingivalis* and significantly reduces coupled bone formation (Behl *et al.* 2008). Thus, altering inflammation alone significantly reduced the amount of bone formation that occurred following resorption. Moreover, application of TNF α or IL-1 β in vivo causes bone resorption and inhibits the coupling process by reducing new bone formation (Bertolini *et al.* 1986; Nguyen *et al.* 1991). It is possible, therefore, that inflammation induced by periodontal pathogens stimulates osteoclastogenesis and bone resorption and also affects bone by limiting the coupling process and reparative bone formation.

There are many instances in which inflammation is linked to uncoupling of bone formation and resorption. One of the most prevalent examples, osteoporosis, is

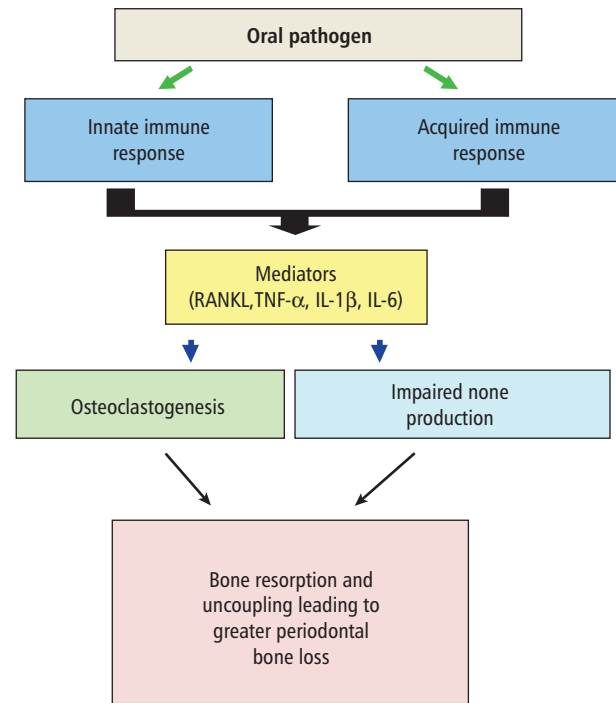


Figure 28.3 Uncoupling is a component of net periodontal bone loss. Oral pathogens stimulate an innate and adaptive immune response that leads to the production of mediators that stimulate bone resorption. In addition, inflammatory mediators are capable of suppressing bone formation, which is programmed to occur after bone resorption. The bone resorption without coupled bone formation leads to net bone loss and formation of an osseous lesion in the periodontium.

characterized by bone resorption that is not followed by adequate bone formation. A potential mechanism for osteoporosis was demonstrated by findings that net loss of bone mass in osteoporosis involves the action of cytokines such as IL-1 or TNF (Weitzmann & Pacifici 2006). More recently, reports indicate that inflammation affects osteoblasts directly and contributes to osteoporosis (Chang *et al.* 2009). When the transcription factor NF- κ B is specifically deleted from osteoblasts, the capacity to form bone is enhanced in ovariectomized mice that would otherwise develop osteoporosis. Interestingly, osteoclastogenesis and osteoclast activity were not affected. In contrast, wild-type ovariectomized mice had significantly impaired coupled-bone formation and osteoporosis. Again, interestingly, NF- κ B affected bone formation by downregulating Fos-related antigen-1 (Fra-1), a transcription factor that coordinates gene expression involved in bone matrix production. Taken together, these experiments demonstrate that inflammation affects bone by reducing the bone forming capacity and that a primary defect in ovariectomy-induced osteo-

porosis is due to the impact of inflammation on osteoblastic cells.

A similar relationship between inflammation and uncoupling has been demonstrated for rheumatoid arthritis. Inflammation destroys the joints induces resorption in the peri-articular bone, which is also characterized by a net loss of bone caused by inadequate coupled bone formation (Walsh *et al.* 2009). Like ovariectomy-induced osteoporosis, TNF α has been implicated in uncoupling associated with arthritic joints as demonstrated by the formation of these lesions in transgenic mice that overexpression of TNF α (Keffer *et al.* 1991). A potential mechanism for uncoupling caused by TNF α has been proposed to involve Dickkopf-1 (DKK-1) (Diarra *et al.* 2007). TNF α stimulates DKK-1, which inhibits bone formation by downregulating the WNT pathway. Thus, TNF α through DKK-1 may promote bone resorption and also inhibit bone formation.

There are a number of mechanisms through which inflammatory mediators may limit bone formation. They may reduce the number of osteoblasts or their precursors by stimulating apoptosis, limiting proliferation, reducing differentiation, or negatively affecting osteoblast activity (Frost *et al.* 1997; Jilka *et al.* 2007; Ghali *et al.* 2010). Inflammatory cytokines may directly or indirectly stimulate osteoblast or osteoblast precursor apoptosis (Tsuboi *et al.* 1999). Osteoblastic cells are particularly sensitive to apoptosis induced by exposure to combined cytokines of the innate and adaptive immune responses in vitro and in vivo (Kuzushima *et al.* 2006; Behl *et al.* 2008). The pro-apoptotic transcription factor, forkhead box-O1 (FOXO1), appears to play a prominent role in cytokine-induced apoptosis in osteoblastic cells (Behl *et al.* 2008). Similarly, prolonged inflammation caused by diabetes mellitus appears to affect bone coupling negatively by stimulating death of bone-lining cells (Al-Mashat *et al.* 2006; Liu *et al.* 2006).

Inflammatory cytokines have also been shown to have other inhibitory effects on osteoblastic cells. High or prolonged levels of TNF α inhibit proliferation of osteoblastic cells. TNF α also reduces osteoblast differentiation by inhibition of runt-related transcription factor 2 (Runx2; Gilbert *et al.* 2002, 2005). TNF α reduces collagen production and alkaline phosphatase activity in osteoblasts (Centrella *et al.* 1988). In addition, production of noncollagen bone matrix proteins is inhibited by pro-inflammatory cytokines (Taichman & Hauschka 1992). Moreover, specific inhibition of TNF significantly improves bone formation following *P. gingivalis*-induced bone resorption (Liu *et al.* 2006) and enhances bone morphogenetic protein 2 (BMP-2)-induced bone formation in vivo (Eguchi *et al.* 2009). Thus, inflammatory

cytokines have multiple mechanisms through which they could limit new bone formation and interfere with bone coupling. This could substantially affect periodontal disease progression by limiting the amount of reparative bone formation following an episode of periodontal bone resorption.

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Clinical correlate: endodontic lesions

Matthew DiAndreth and Hongjiao Ouyang

Pulpal and periapical pathosis are caused by oral bacteria and/or their by-products. Kakehashi and colleagues (1965) demonstrated that pulp exposure in gnotobiotic (germ-free) rats led to low-grade pulp inflammation and calcification. In contrast, the same procedure in conventional rats resulted in pulp necrosis and abscess formation (Kakehashi *et al.* 1965). Under normal circumstances, pulp tissues are protected by surrounding enamel, dentin, and cementum. Any carious, mechanical, or chemical irritants that damage the enamel, dentin, or cementum can expose the pulp tissues to oral bacteria and their toxins. After actual pulp exposure occurs, bacteria can invade and colonize in the pulp tissues. Pulpal tissues may stay inflamed for an extended time before they eventually undergo necrosis or become necrotic quickly after pulp exposure to the oral cavity. Subsequently, the bacteria, their toxins, and/or the irritants from the necrotic pulpal tissues can spread into periapical tissues and induce periapical inflammation and tissue destruction.

Among the many etiological factors that can cause pulp exposure is dens evaginatus, a rare developmental anomaly that is predominant in Mongoloid populations, for example Asian populations (Hill & Bellis 1984). *Dens evaginatus* refers to an extra cusp or tubercle that protrudes from the lingual surface of anterior teeth and the occlusal surface of posterior teeth (Hill & Bellis 1984). It often occurs bilaterally (Ju 1991). Since the tubercles are easily fractured during chewing and biting activity, dens evaginatus often challenges practitioners with its frequent pulpal complications.

In addition, trauma to teeth could also involve pulpal tissues and lead to periapical pathosis. Injuries often

occur in the 7- to 12-year-old age group (Andreasen & Andreasen 1994). The bacteriological studies of Moller and colleagues (1981) and Bergenholtz (1974) demonstrated that pulpal tissues in traumatized teeth without apical lesions were aseptic, whereas those with periapical pathosis had bacterial contamination. These studies highlight the role of bacterial infection in trauma-induced pulp and periapical pathosis.

Endodontic treatment (root canal treatment) that consists of both mechanical and chemical debridement aims at thoroughly debriding the root canal system of bacteria, their by-products, and infected pulpal tissues as well as appropriately shaping the canal system to receive a three-dimensional hermetic filling of the entire root canal space (Schilder 1974). Once pulpal irritants are removed, healing of periapical lesions in the form of either regeneration or repair ensues. Therefore, endodontic treatment often results in resolution of pulpal and periapical pathosis in a highly predictable manner (Eriksen 1991).

Case presentation

Case A

Mr. T., a 21-year-old Asian male in good general health, presented to the Graduate Endodontics Clinic at the University of Michigan, School of Dentistry. His chief complaint was “I have pus in my mouth.” He reported an unknown drug allergy, no previous dental treatment, and no history of trauma.

A comprehensive oral examination that included extraoral, intraoral, and radiographic assessments was performed. The extraoral examination revealed no

swelling in the facial and neck area and no lymphadenopathy. An intraoral evaluation revealed an active sinus tract in the attached gingival tissue approximately 5 mm below the gingival margin of tooth 20 (Figure 29.1A). The periodontal ligament (PDL) probing of tooth 20 was within normal limits. A periapical (PA) radiograph was exposed with gutta percha tracing. The PA radiograph revealed an extensive radiolucent area around the apex of tooth 20 that extended upward along the distal side of the root and also involved the mesial aspect of the mesial roots of tooth 19 (Figure 29.1D). The inserted gutta percha dropped into the PA lesion (Figure 29.1D). Pulp vitality tests were performed on teeth 19, 20, and 21. Tooth 20 lacked response, while teeth 19 and 21 exhibited normal responses to the pulp vitality tests. In addition, tooth 20 displayed mild sensitivity to the percussion and palpation tests, while teeth

19 and 21 had normal responses to both tests. Since the PA lesion was J-shaped, Tooth Slooth tests (Professional Results, Inc., Laguna Niguel, CA, USA) were also performed on teeth 19, 20, and 21. The results excluded the possibility of fracture on these teeth. Based on the clinical evaluations described here, the clinical diagnoses for tooth 20 were necrotic pulp and suppurative periapical abscess. However, the cause for the necrotic pulp was puzzling, since tooth 20 was neither decayed, nor traumatized, nor periodontally compromised. Careful intraoral examination revealed that tooth 20 possessed an unusual “extra cusp” in the center of its occlusal surface, which was severely worn (Figure 29.1C). It became clear that tooth 20 had a severely worn dens evaginatus resulting in compromised structural integrity of the crown and consequent pulp infection and suppurative periapical abscess.

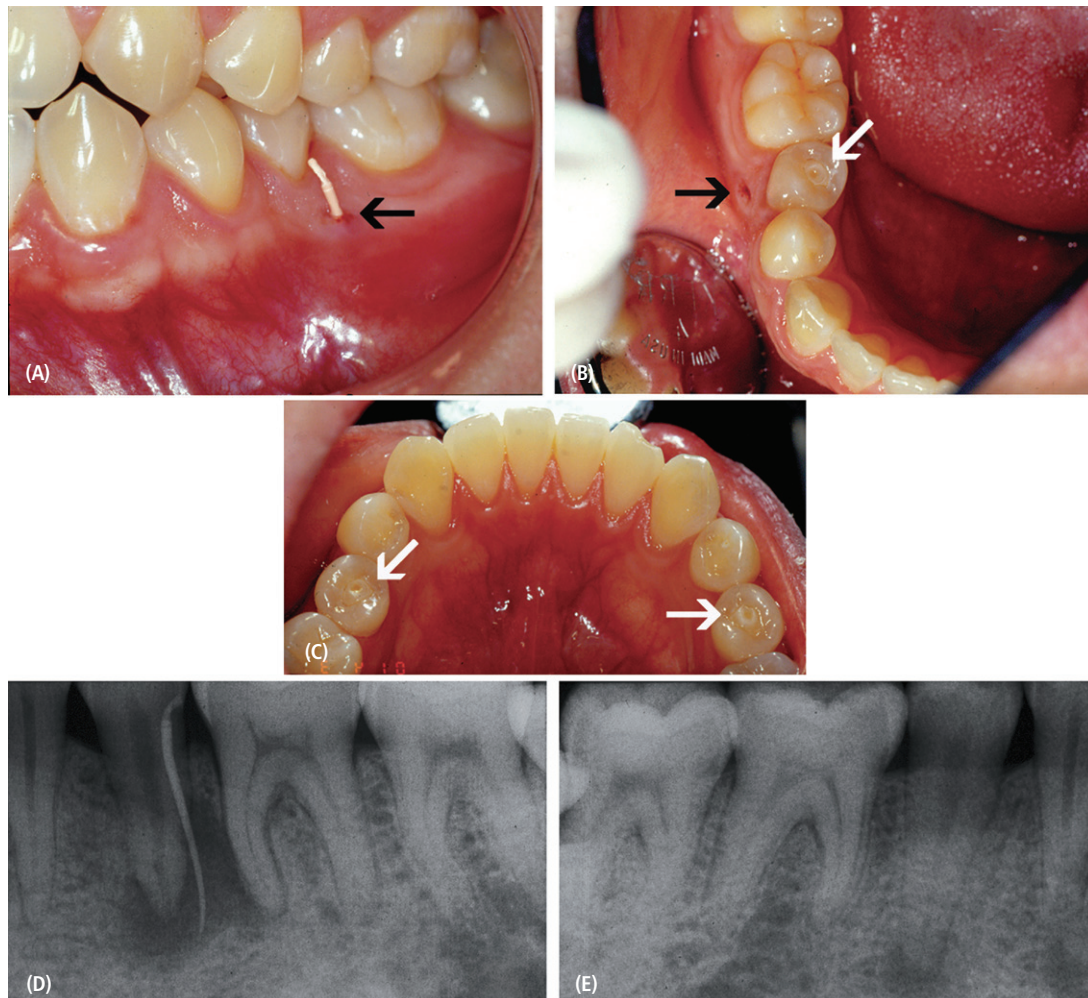


Figure 29.1 A. Buccal view of the pre-operative clinical presentation of the sinus tract (black arrow) of tooth 20 with a gutta percha inserted. B. Occlusal view of the pre-operative clinical presentation of the dens evaginatus (white arrow) and the sinus tract (black arrow) of tooth 20. C. Occlusal view of the preoperative clinical presentation of bilateral dens evaginatus (white arrows) on teeth 20 (right) and 21 (left). D. Pre-operative PA radiograph of tooth 20. E. Pre-operative PA radiograph of tooth 29.

Because dental anomalies like dens evaginatus usually occur bilaterally (Ju 1991), tooth 29 was also examined. Interestingly, tooth 29 also had a worn extra cusp in the center of its occlusal surface and a partially healed sinus tract in its attached gingival tissue (Figure 29.1B–C). The tooth displayed no response to the pulp vitality test and mild hypersensitivity to the percussion and palpation tests. In contrast, adjacent teeth 28 and 30 exhibited normal responses to these tests. Periodontal probing of tooth 29 was within normal limits. A PA radiograph indicated a radiolucent area around the apex of the root (Figure 29.1E). The clinical diagnoses were necrotic pulp (due to worn dens evaginatus) and chronic periapical periodontitis.

The root canal treatment was performed for teeth 20 and 29 sequentially. The patient was administered 1.5 carpules of 2% lidocaine with 1:100K epinephrine. A rubber dam was placed and the coronal access was made with a #4 round bur and a pulp shaper bur on the occlusal surface. Endodontic working length was obtained using a Root ZX Apex Locator (J. Morita USA, Irvine, CA) and confirmed by a periapical radiograph (not shown in the figures). The teeth were prepared with the K-files, Tulsa Profile Series 29 rotary files in combination with sodium hypochlorite rinses in a crown-down fashion. Upon the completion of canal preparation, no pus was observed draining through the canal. A master gutta percha cone was fitted to length, which was confirmed by radiography (not shown in the figures). Obturation was performed using a warm vertical technique with Roth's sealer. A cotton pellet and a temporary restoration of zinc oxide eugenol (ZOE) were placed, sealing the access opening. A final radiograph was taken to confirm the completion of endodontic treatment (Figure 29.2).

At the six-month recall visit, Mr. T. reported a lack of symptoms. Clinical examination revealed final compos-

ite restoration of teeth 20 and 29 and completely healed sinus tracts for both teeth. Periodontal probing was within normal limits, and both teeth responded normally to percussion and palpation tests. Radiographic examination revealed partial healing of the periapical lesion of both teeth. Teeth 20 and 29 remained asymptomatic one year post treatment. Radiographic examination revealed complete resolution of the PA pathosis for both teeth (Figure 29.3).

Case B

Miss H., a 12-year-old Caucasian female, presented to the Precision Endodontics Clinic with pain associated with the maxillary left anterior teeth 9 and 10. The pain had been present for two weeks, and she had been taking 250 mg Amoxicillin twice a day for two weeks. Miss H. was also on a Daytrana 20 mg patch per day for attention deficit-hyperactivity disorder (ADHD). She had no other contributory medical history.

She reported traumatizing the maxillary anterior region approximately two years prior to her visit to the endodontics clinic at which time she fractured the incisal edge of tooth 9. Composite bonding was applied to repair the fracture of tooth 9 by her general dentist the week after the trauma. She reported no pain present at that time. Two weeks prior to her visit to the endodontic clinic, the composite was dislodged and tooth 9 was tender to touch.

The intraoral clinical examination revealed a slight swelling in the apical area of both teeth 9 and 10. Periodontal probing was within normal limits with less than a 1° mobility for both the central and lateral incisors. Radiographic examination revealed a large radiolucency associated with the apical region of tooth 10 and distal aspect of tooth 9 (Figure 29.4A). The pulp vitality tests for the lateral incisor were normal and the central incisor did not respond. The central incisor was also percussion

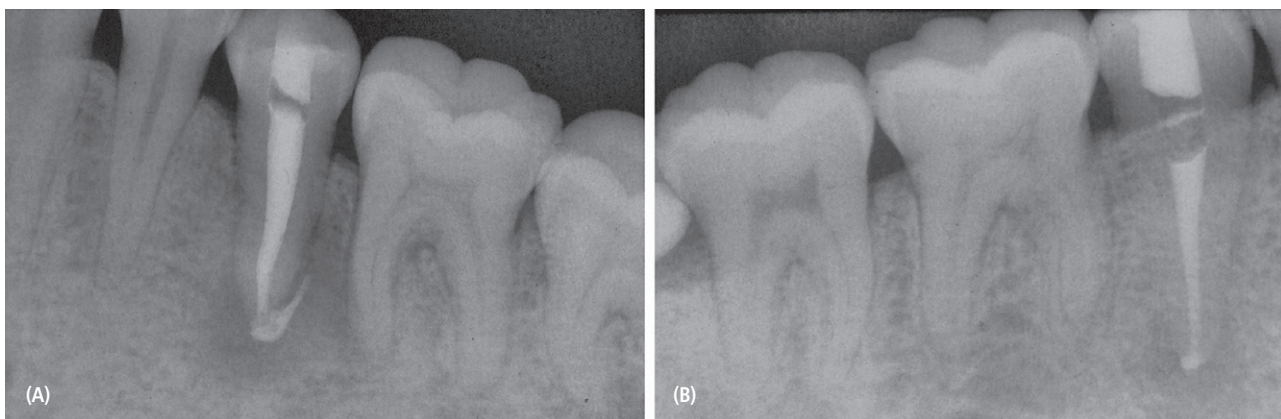


Figure 29.2 PA radiograph of teeth 20 (A) and 29 (B) taken immediately post root canal treatment.

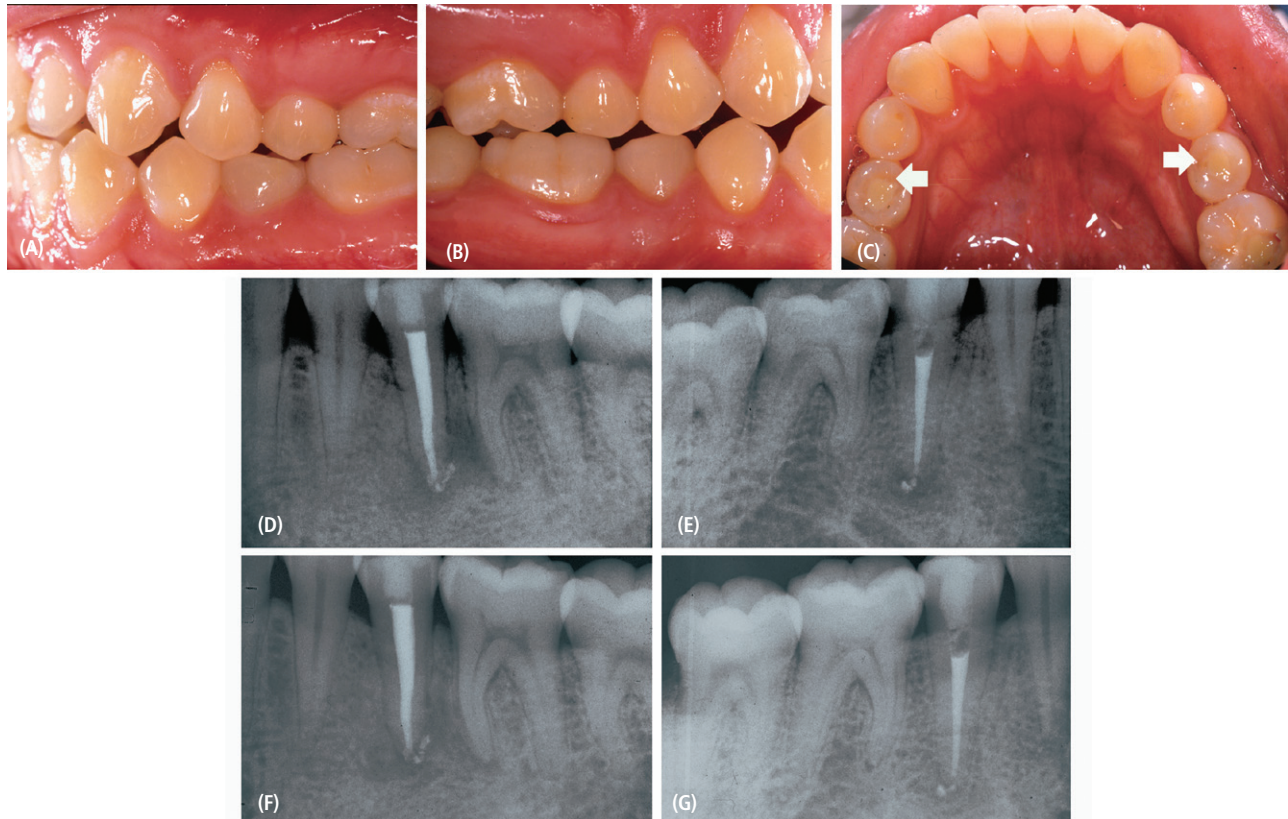


Figure 29.3 A–B. Buccal views of teeth 20 (A) and 29 (B) taken six months post treatment showing complete healing of the sinus tracts. C. Occlusal view taken six months post treatment showing composite restoration (arrow heads) of teeth 20 (right) and 29 (left). D–E. PA radiographs of teeth 20 (D) and 29 (E) taken six months post treatment showing partial resolution of periapical radiolucency. F–G. PA radiographs of teeth 20 (F) and 29 (G) taken one year post treatment showing complete resolution of periapical radiolucency.

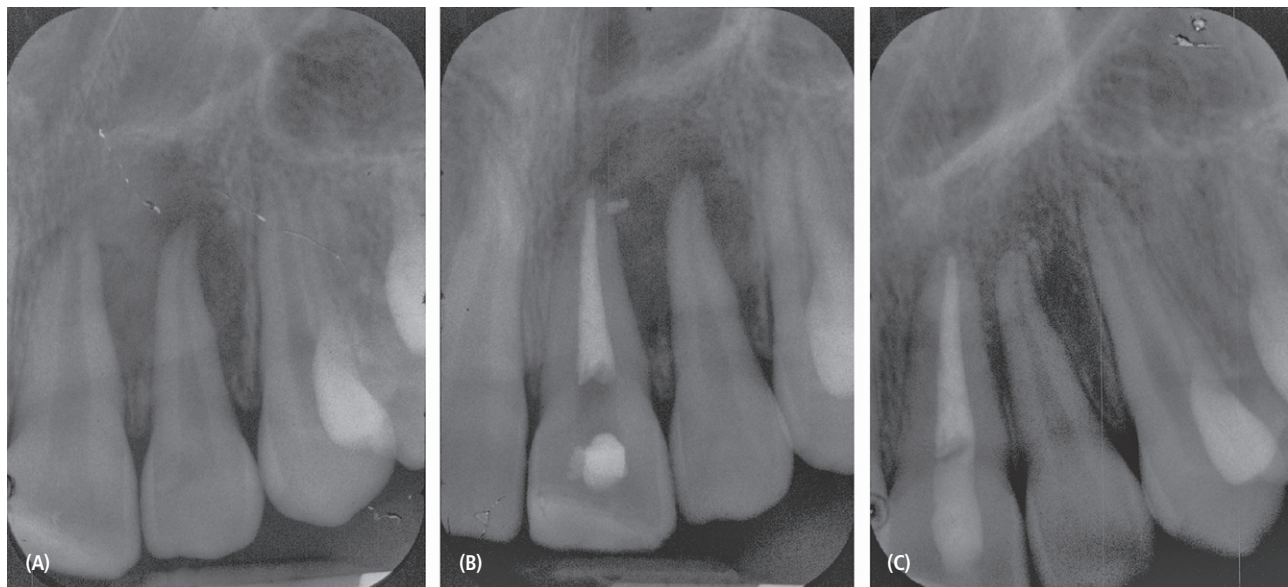


Figure 29.4 A. Preoperative PA radiograph showing the PA radiolucency of teeth 9 and 10. B. Post-endodontic treatment PA radiograph showing completed RCT of tooth 9. C. One year post-endodontic treatment PA radiograph showing resolution of PA radiolucency of teeth 9 and 10.

positive while the lateral incisor was negative. The diagnosis was necrotic pulp and an acute apical abscess associated with tooth 9. Endodontic treatment was performed in a similar fashion, as described in Case A of this chapter (Figure 29.4B). Tooth 10 was determined to be vital and no endodontic treatment was indicated.

One year post treatment, the clinical examination revealed that tooth 9 had been permanently restored with composite. Both teeth 9 and 10 displayed normal mobility, and periodontal probing was also within normal limits for both teeth. Percussion tests were negative on both teeth. Radiographic examination revealed that healing of the periapical lesion of teeth 9 and 10 was complete (Figure 29.4C).

Discussion

It is well established that both the pulpal inflammation and periapical lesions are associated with bacterial infection (Kakehashi *et al.* 1965; Bergenholtz 1974; Moller *et al.* 1981). Both clinical scenarios presented in this chapter support this notion. In Case A, the patient has dens evaginatus, a developmental anomaly that occurs most frequently in mandibular premolars (Oehlers *et al.* 1967). Since the tubercles occurred in the occlusal surfaces of teeth 20 and 29, they were easily chipped away (Figure 29.1B–C). The amount of wear resulted in pulpal exposure to the saliva-borne bacteria and consequent pulp necrosis and periapical pathosis of teeth 20 and 29.

In Case B, trauma of tooth 9 led to pulp exposure to saliva-borne bacteria resulting in pulp necrosis and an acute apical abscess, and dislodging of the incisal composite coincided with the initiation of clinical symptoms, such as tenderness upon palpation and apical swelling. Although tooth 10 was also affected by the previous trauma, it did not develop pulp pathosis since it remained structurally intact. This clinical case confirms the suggestion that bacterial infection plays a critical role in trauma-induced pulpal and periapical pathosis (Bergenholtz 1974).

A comprehensive clinical examination, including bilateral inspection, is critical for correct diagnosis and accurate identification of the affected teeth. In Case A, careful clinical evaluation of the bilateral dentition revealed the presence of dens evaginatus in teeth 20 and 29 as well as consequent pulp and periapical pathosis. Without a comprehensive bilateral clinical assessment of the dentition, it is very likely that tooth 29 would have remained undiagnosed since it was asymptomatic.

In Case B, the periapical radiographic examination revealed that the radiolucency was mainly associated with tooth 10. Without careful pulp vitality and percussion tests, tooth 10 could have been mistakenly held

responsible for the disease condition. At one year post treatment, the root canal treatment of tooth 9 led to complete resolution of the periapical pathosis of both maxillary left incisors, confirming that tooth 9 was the affected tooth. This case demonstrates that although radiographic evaluation serves as a diagnosis and treatment aid, the dental practitioner should never exclusively rely on radiographic images to make pulpal and periapical diagnoses. In summary, both cases A and B demonstrate the importance of comprehensive endodontic examinations in providing accurate endodontic diagnosis and successful endodontic treatment.

Conclusion

Bacterial infection is the critical etiological factor responsible for pulpal and periapical pathosis. Resolution of pulp and periapical tissue infection and healing of periapical tissues are highly predictable with root canal treatment. Careful endodontic evaluation is vital for correct diagnosis and identification of the affected tooth or teeth, as well as the success of endodontic treatment.

Summary

Pulp and periapical pathosis is induced by bacteria infection. The loss of enamel, dentin, or cementum may lead to bacterial infection of pulpal and periapical tissues and consequently pulpal and periapical pathosis. There are a variety of causes that can compromise the structure integrity of enamel, dentin, or cementum. These include caries, trauma, and developmental anomalies. Case A presents an example in which dens evaginatus of teeth 20 and 29 led to necrotic pulp and periapical pathosis. Case B describes trauma-induced necrotic pulp and acute periapical abscess of tooth 9. Comprehensive endodontic examination, root canal treatment, and subsequent timely crown restoration resulted in successful resolution of both pulpal and periapical infection and achieved periapical bone healing in both cases.

Acknowledgments

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Biomechanics of teeth in bone: function, movement, and prosthetic rehabilitation

Susan W. Herring

The tooth–bone interface is a mechanical linkage

The tooth–bone interface consists of the cementum-covered root, the periodontal soft tissues, and the bony alveolar socket. Each of these structures is a complex of cellular and matricial constituents, variably arranged and geometrically irregular. The three major components are physically linked via a collagenous network, which includes not only the periodontal ligament (PDL) proper, but also Sharpey's fibers that can penetrate the root as far as the dentin (Ho *et al.* 2007) and can traverse the entire interdental septum of alveolar bone (Cohn 1975). Functionally, the teeth are tools for manipulating and breaking down objects in the mouth, and thus the tooth–bone interface is a mechanical device. The arrangement of the interface as two hard parts separated by a soft part is not a vertebrate inheritance, but a mammalian invention that accompanied the evolution of controlled eruption, precise occlusion, and chewing. Very few nonmammalian vertebrates even have cement-covered roots in sockets (Gaengler 2000; Cisneros *et al.* 2011), and those that do have mineralized periodontal ligaments (McIntosh *et al.* 2002; Luan *et al.* 2009) and hence, must be relatively inflexible.

In mammals, the flexibility of the attachment apparatus serves the short-term function of bearing occlusal loads and transmitting them to the supporting bone while, at the same time, providing protection against sudden impacts. Long-term, this adaptable apparatus furnishes the cellular, sensory, and vascular elements needed for tooth position to be adjusted by eruption (Gaengler 2000), by drift, or in response to applied loads. The most obvious physiological correlate of the adapt-

ability of the periodontium is a remarkably high turnover rate of collagen and bone (Sodek & Ferrier 1988; Sodek & McKee 2000), leading Berkovitz to characterize the periodontal soft tissues as almost fetal in their behavior (Berkovitz 2004). Similarly, bone remodeling in the jaws takes place at an elevated juvenile rate even in adults (Huja *et al.* 2006; Huja & Beck 2008), a finding that, it has been speculated, suggests continual repair of micro-damage inflicted by occlusal loading (Burr & Allen 2009).

This chapter is organized at progressive levels of complexity. The most basic level is the material properties of the individual components featured in the “Mechanical Properties” section. The next level, “Loading of Teeth and Jaws in the Mouth,” considers how these components work together and their collective functional behavior. The third level, “Modeling and Remodeling in Response to Mechanical Strain,” incorporates time and discusses how the tooth–bone interface adapts to prevailing conditions. The fourth level moves to clinical problems, specifically how periodontal mechanics are altered by tooth loss. The chapter concludes with a consideration of future directions.

Mechanical properties of periodontal constituents

Representative data on modulus are summarized in Table 30.1. A caveat of this tabulation is that fresh, intact human tissue cannot be obtained in any quantity, so data are derived from other species. The tooth–bone interface is a product of evolution, and the periodontia of different teeth in different mammals must be understood on their own terms before being extrapolated to the human

Table 30.1 Measured material properties of the tooth–bone interface.

	Modulus ^a	Specimens	Test
Jaw cortical bone	11.3–18.9 GPa (E)	Colobine monkey mandible sections	Microindentation, dry (Daegling <i>et al.</i> 2011)
	9.2–25.8 GPa (E)	Baboon mandibular cortex	Ultrasound (Wang <i>et al.</i> 2010)
	12.7–25.2 GPa (E)	Human mandibular cortex, including alveolar	Ultrasound (Schwartz-Dabney & Dechow 2003)
	12.5–16.5 GPa (E _r)	Dog mandibular cortex, alveolar region	Nanoindentation wet (Huja <i>et al.</i> 2007)
	6.9–18.7 GPa (E)	Human maxillary cortex, including alveolar	Ultrasound (Peterson <i>et al.</i> 2006)
	9.9–15.7 GPa (E)	Macaque monkey maxillary cortex	Ultrasound (Wang & Dechow 2006)
	9.2–11.4 GPa (E _r)	Dog maxillary cortex, alveolar region	Nanoindentation wet (Huja <i>et al.</i> 2007)
Alveolar bone	0.2–9.6 GPa (E _r)	Human mandible, molar alveolus	Nanoindentation wet, (Ho <i>et al.</i> 2010)
	9.0–15.8 GPa (E)	Colobine monkey mandibular alveoli	Microindentation, dry (Daegling <i>et al.</i> 2011)
	10.7–12.7 GPa (E _r)	Dog mandibular alveoli	Nanoindentation wet (Huja <i>et al.</i> 2007)
	8.4–12.1 GPa (E _r)	Dog maxillary alveoli	Nanoindentation wet (Huja <i>et al.</i> 2007)
Bone–ligament enthesis	0.1–1.0 GPa (E _r)	Human mandibular molar alveolus	Nanoindentation (Ho <i>et al.</i> 2010)
Ligament			
<i>Overall</i>	0.45–1.34 MPa (E)	Rat, hamster, rabbit incisor sections	Intrusion, extrusion, regional variation (Chiba <i>et al.</i> 1990; Komatsu <i>et al.</i> 1998)
	1–6 MPa (E)	Mouse incisor sections	Extrusion, regional variation (Komatsu <i>et al.</i> 1998)
	0.22 ± 0.06 MPa (G*)	Pig incisor sections	Dynamic shear (Tanaka <i>et al.</i> 2006)
	0.14–0.19 MPa (G*)	Pig molar sections	Dynamic shear, regional variation (Tanaka <i>et al.</i> 2007)
	1–8 MPa (E)	Cow incisor and molar sections	Transverse tension (Pini <i>et al.</i> 2004)
	5–19 MPa (E)	Cow molar sections	Dynamic tension, variable rate (Sanctuary <i>et al.</i> 2005)
<i>Initial only</i>	0.012 MPa (G)	Pig premolar	Intrusion, optical (Natali <i>et al.</i> 2007)
	0.04 ± 0.02 MPa (E)	Human incisor	Translation, optical (Liu <i>et al.</i> 2011)
	0.1 MPa (E)	Pig molar	Intrusion, optical (Qian <i>et al.</i> 2009)
	0.05 ± 0.02 MPa (E)	Pig primary molar	Intrusion (Ziegler <i>et al.</i> 2005)
	0.15 ± 0.07 MPa (E)	Rat molar	Intrusion (Kawarizadeh <i>et al.</i> 2003)
<i>Late only</i>	0.8 MPa (E)	Pig molar	Intrusion, optical (Qian <i>et al.</i> 2009)
	0.16 ± 0.03 MPa (E)	Human incisor	Translation, optical (Liu <i>et al.</i> 2011)
	0.18 ± 0.12 MPa (E)	Pig primary molar	Intrusion (Ziegler <i>et al.</i> 2005)
	0.60 ± 0.18 MPa (E)	Rat molar	Intrusion (Kawarizadeh <i>et al.</i> 2003)
Cementum–ligament enthesis	0.1–0.6 GPa (E _r)	Human molar	Nanoindentation (Ho <i>et al.</i> 2010)
Cementum–dentin junction (CDJ)	2–4 GPa (E)	Human molar	Nanoindentation (Ho <i>et al.</i> 2007)
Dentin	10–25 GPa (E _r)	Human molar	Nanoindentation (Ho <i>et al.</i> 2010)
	17–50 GPa (E)	Human molar	Viscoelastic, regional variation (Balooch <i>et al.</i> 2004)
	21–27 GPa (E)	Bovine incisor	Nanoindentation (Inoue <i>et al.</i> 2009)

^aE: elastic modulus; E_r: reduced elastic modulus; G: tangent shear stiffness; and G*: complex dynamic shear modulus. For details and information on techniques used, see references in table.

condition. For example, dynamic testing of bovine molars has shown the surprising result that there is no difference between stiffness during intrusion and that during extrusion (Sanctuary *et al.* 2006); however, bovine teeth are loaded primarily in the horizontal plane by grinding mastication, and such a conclusion should not be extended to human teeth for which vertical loading is probably more important.

Methods for deriving material properties

The material properties of the periodontal components require empirical measurement, which is difficult because of small size, structural complexity, and viscoelasticity. Typically the specimens must be sectioned. The mineralized tissues are most often characterized using micro- or nano-indentation, preferably in hydrated samples (Ho *et al.* 2010). The periodontal ligament is tested wet, usually in tension or shear, by manipulating the hard parts to which it is attached. The references cited in Table 30.1 employed a variety of innovative techniques to explore its properties.

Alveolar bone

Because alveolar bone turns over rapidly and, therefore, is not well mineralized, one might expect it to be rather flexible. This suspicion was confirmed by indentation testing of wet specimens, which produced values of 0.2 to 9.6 GPa (Ho *et al.* 2010) in contrast to the values of 18 to 20 GPa from postcranial cortical bone that are commonly used in simulations. Lower moduli in mandibular alveolar bone as compared to those of the corpus also characterize dry specimens in most studies (Daegling *et al.* 2011). Numerical models predict, and *ex vivo* measurements confirm, that alveolar bone bends readily in response to occlusal loading (Popowics *et al.* 2009; Qian *et al.* 2009; Yeh *et al.* 2010).

One of the most interesting aspects of alveolar bone is the difference between the maxilla and the mandible, even though the upper and lower jaws obviously bear the same magnitude of occlusal loading. Canine mandibular cortical bone is harder than maxillary cortical bone (Huja *et al.* 2007). In addition, the trabecular bone around the roots of mandibular teeth shows a strong mesiodistal orientation (Figure 30.1), which has been interpreted as aligned with tensile stress that occurs along the alveolar edge when occlusal force bends the mandible in cantilever fashion (Hildebrand 1988). In contrast to the overall thick cortex of the mandible, maxillary cortical bone is thin except for structural pillars leading up to the frontal and zygomatic bones, and maxillary trabecular bone appears isotropic. The mandible typically has a higher remodeling rate than the maxilla (Huja & Beck 2008; Meta *et al.* 2008) and is more vulner-

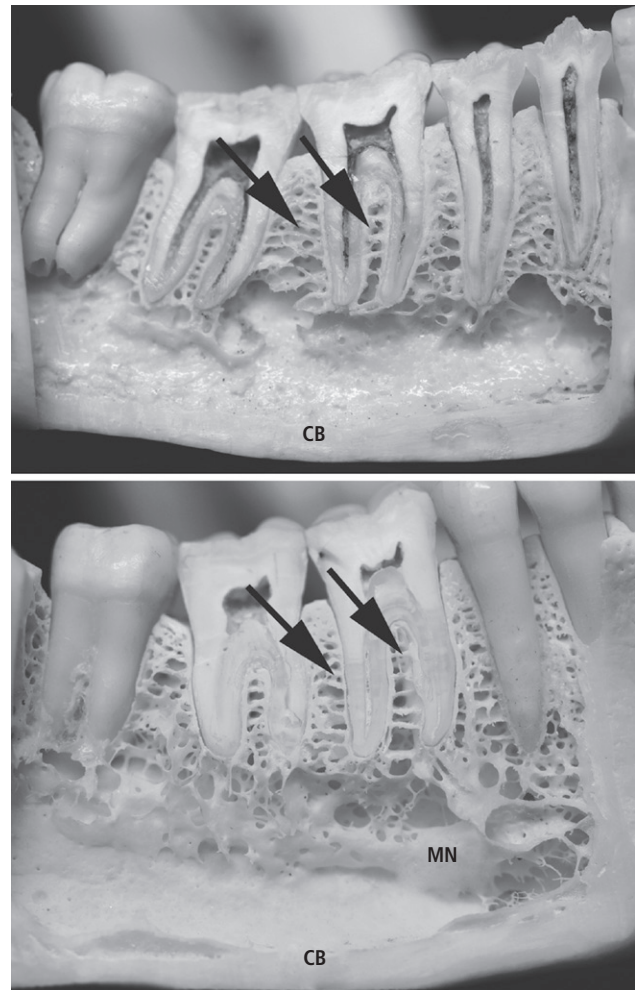


Figure 30.1 Two human mandibles sectioned parasagittally to show the mesiodistal alignment of trabeculae (arrows) between teeth and in the interradicular area. This arrangement may resist tensile stress imposed by bending, while the thick cortical bone (CB) of the lower border resists compressive stress (Hildebrand 1988). The maxilla, which is supported by the remainder of the craniofacial skeleton and is not bent, has randomly arranged trabeculae and a generally thinner cortex. MN: canal and opening for the mental nerve.

able to bisphosphonate osteonecrosis (Ruggiero *et al.* 2004; Danneman *et al.* 2007). The most common explanation for all these differences is that the entire maxilla is braced by other craniofacial bones, whereas the mandible is supported only at the back and hence is more subject to bending and torsion. The stresses resulting from this more vulnerable condition would lead to more obvious adaptations to resist loading and greater turnover to replace higher levels of damaged bone.

Periodontal ligament

Because the root and the bony socket are much stiffer than the intervening soft tissue, loads deform this space

much more than the other components (Chattah *et al.* 2011). The soft tissue compartment includes glycosaminoglycans (GAGs) and fluid that are the primary locus of viscoelasticity for the tooth–bone interface as a whole. Tears and avulsions take place here as well. Specimen preparation usually precludes the preservation of the fluid elements, so measurements of the material properties of soft tissues concentrate on the periodontal ligament, specifically the principal fibers that occupy most of the root area. The principal fibers are arranged in “hammock” fashion, suggesting that they bear tensile load when the tooth is intruded, but because they show crimping (Sloan & Carter 1995), their stiffness is initially low, rising only when the crimps have been pulled straight. This effect, typical of collagenous tissues, causes nonlinearity of the stress–strain curve, and therefore, many studies have considered the periodontal ligament as bilinear (denoted as initial and late phases in Table 30.1). What is not known, however, is whether the late phase is ever reached during normal function. If so, then the ligament could partially act as a spring, returning the energy used for the initial stretch (Komatsu *et al.* 2007). Another significant point is that although the periodontal ligament is usually tested at slow strain rates, its viscoelasticity results in much greater stiffness at faster strain rates, which more closely resemble chewing (Sanctuary *et al.* 2005).

The mechanical properties of the periodontal ligament in situ have been measured in a variety of species using a variety of methods. Nevertheless, the results are consistent in showing that the ligament is anisotropic and has stiffness in the range of 0.01–19.00 MPa (Table 30.1). Yet the values used in numerical simulations are often higher, causing one sensitivity study to run computations using values up to 1750 MPa (Panagiotopoulou *et al.* 2011).

In general, the attachment areas of ligaments (entheses) are zones of weakness. The periodontal ligament is no exception, as indicated by the fact that about half of the root surface of traumatized and extracted teeth is completely denuded (Haas *et al.* 2008). Presumably, the same thing occurs at the bony surface, although, understandably, no observations have been reported. High-resolution imaging and indentation studies show that the cemental and bony entheses become increasingly mineralized and stiff at the tissue interface (Ho *et al.* 2010), a stress-relieving adaptation.

Cementum

Cementum is a bone-like intermediary connecting the ligament to the root dentin. Combining chemical and mechanical analyses with high-resolution imaging, Ho and colleagues have shown an intricate arrangement of

collagen fibers that fan out at both ends (Ho *et al.* 2010). These attaching regions had lower stiffness (E , 1–4 GPa) than the cementum proper (4 to 7 GPa), reflecting higher GAG content and lower mineralization, indicating that this junction also could be stress relieving (Ho *et al.* 2010).

Overview

This short summary and the data in Table 30.1 lead to several conclusions. First, while modulus values for cortical bone in the jaws fall in the low end of the range for postcranial cortical bone, alveolar bone is distinctly more flexible. Second, although measured values for periodontal ligament stiffness vary, they are uniform when compared with the values used in numerical simulations (Panagiotopoulou *et al.* 2011). Third, the soft tissues behave differently for rapid versus sustained loading. For rapid loading like mastication, the response is elastic and the tissues relatively stiff, but for sustained loading like orthodontic treatment, viscosity prevails. Fourth, when the interface fails and trauma intrudes or avulses a tooth, it seems likely that the entheses of the ligament constitute a weak link requiring adaptation in the form of graded material properties at the attachments of the ligament to hard tissue (Ho *et al.* 2010).

Loading of teeth and jaws

Most of the loads encountered by the tooth–jaw interface are dynamic, arising from muscle contraction during feeding, speech, and facial expression. The largest loads are probably those from occlusal force during the power stroke of mastication, but these are much less frequent than the smaller forces from other activities (de Jong *et al.* 2010).

Functional morphology of the tooth–bone interface

Although the variations and morphology of dental roots are well known, little attention has been given to functionally significant features such as the surface area available for attachment of periodontal ligament fibers or the effect of shape variations on mechanics. From a comparative perspective, it is clear that crown characteristics need not be well correlated with root morphology. Figure 30.2 illustrates this point with two species of bat, which have similar crown area but vastly different root area. Algorithms now available for CT scans have made quantitative measurements possible. These have revealed remarkable reductions of root surface area in modern humans compared to earlier *Homo sapiens* (Kupczik & Hublin 2010). Our more gracile and separated roots imply a lesser ability to resist occlusal loading than those

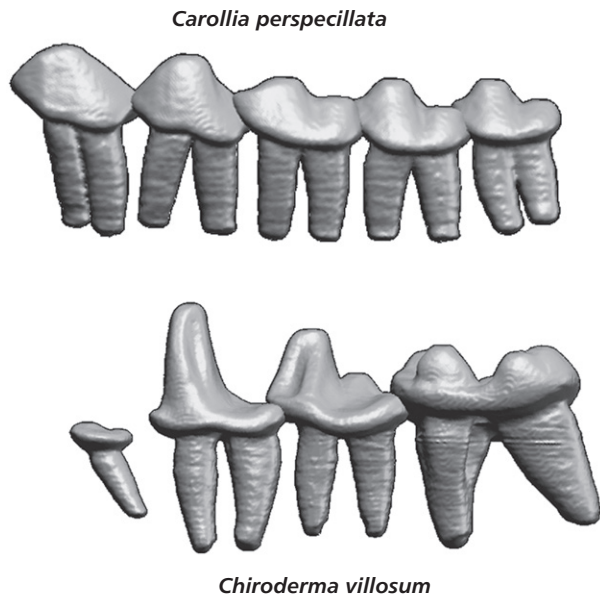


Figure 30.2 Micro-CT reconstructions comparing the teeth of two fruit-eating bats. *Chiroderma villosum* has larger roots relative to crowns than *Carollia perspicillata* and a much higher bite force (Freeman & Lemen 2010). (Figure is courtesy of Casey Self, University of Washington.)

of our near ancestors, presumably related to our softer diets.

Another functionally significant, but unstudied, feature is the width of the periodontal space, which constrains tooth mobility. Major regional and interspecies differences exist. For example, the width of the periodontal space in bovine molars averages 551 μm , roughly three times larger than in humans (Bosshardt *et al.* 2008), suggesting that tooth mobility is much greater in cattle. Number and angulation of the roots undoubtedly influence mobility as well. In the future, micro CT scans will enable accurate depictions of complex roots and sockets.

The periodontal ligament has been hard to quantify in terms of fiber numbers, but orientation is described in detail in most textbooks. In general, the fibers are wavy and many areas are plexiform with interwoven branches. This morphology suggests that the collagen fibers become important in bearing load only when they have been straightened out by the movement of the tooth within the socket (initial phase; Table 30.1). The coronal fibers are braided with gingival collagen and hence connect adjacent teeth and unite the bone, tooth, and gingiva. Apical fibers connect the root directly to the socket and are appropriately oriented to resist extrusion. The principal fibers that occupy most of the root are obliquely arranged such that they would be tensed when the tooth was intruded. In transverse section, the liga-

ment is cruciform, an arrangement interpreted to signify resistance to rotational loads (Sloan & Carter 1995). Perhaps the best evidence that periodontal ligament fiber orientation is adapted to prevalent loading conditions is that specializations are seen in specialized teeth (Spence 1978; Staszyk *et al.* 2006).

The fluids of the periodontal space include not only the “ground substance,” consisting of hydrophilic GAGs largely bound to the collagen fibers (Watanabe & Komatsu 1997), but also an abundant vasculature. Vessels are protectively situated in between collagen bundles and connected liberally with vessels in the alveolar bone. This vascularity facilitates the high levels of turnover that characterize the periodontium and serves to mitigate the focal necroses (hyalinized zones) caused by orthodontic tooth movement. In addition, it is presumed that the vessels have a role in mechanics. Arterial blood pressure is important in determining the position of unloaded teeth (Myhre *et al.* 1979).

The living attachment apparatus

Mobility of teeth relative to their sockets is the only mechanical feature of the tooth–bone interface that is amenable to measurement. Laborious studies of the twentieth century showed that tooth mobility varies depending on the direction of force, apparently under the control of the periodontal ligament (Heners 1974), and that the alveolar bone deforms readily even for relatively low loads (Picton 1965) but not always in the expected direction (Mühlemann 1968). For brief loads, the periodontal fluid system can stabilize the root, but over the longer term, fluids are redistributed (Mühlemann 1968). Simulated mastication was noted to produce progressive intrusion of teeth which recovered only slowly afterward, presumably in relation to fluid movement (Picton & Wills 1978). More recent studies have used magnetic sensors (Yoshida *et al.* 2001) and photogrammetry (Göllner *et al.* 2009), but they share the inability of early studies to document tooth mobility during normal function. We still have no idea how far, and in what direction, the tooth is allowed to move within its socket during normal function. However, such measurements are now possible, at least for animals. Figure 30.3 illustrates an ultrasound method for recording the position of a tooth root relative to alveolar bone in an awake pig chewing naturally.

Another feature amenable to measurement in animals, but not humans, is how much, and in what direction, the flexible alveolar bone bends. Bone strain could be ascertained in vivo from strain gages bonded to the alveolar bone. As yet, however, studies have measured only the base of the alveolar process (Weijs & de Jongh 1977; Yeh *et al.* 2010) and not the socket itself.

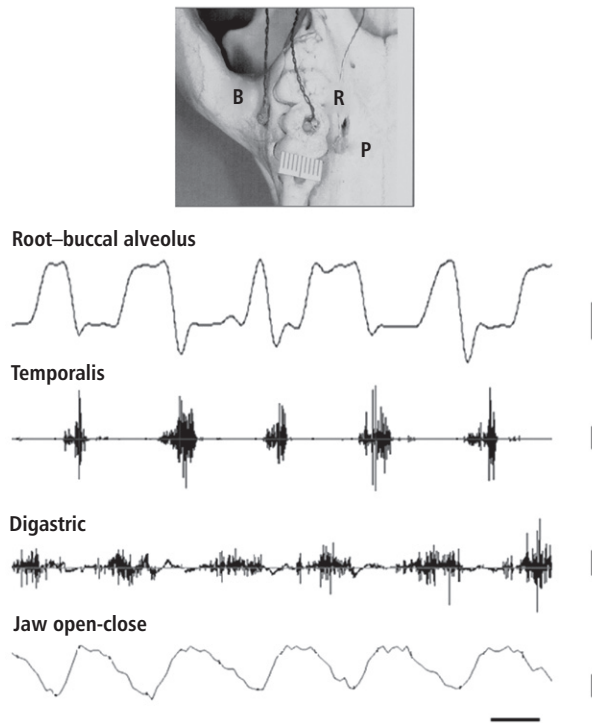


Figure 30.3 Photograph of a dry pig skull showing the placement of crystals that transmit and receive ultrasound signals. Crystals B (buccal) and P (palatal) are in the alveolar bone, and crystal R (root) is inserted into the distal-palatal root of the molar. The crown is then restored. The recording of mastication (from a different pig) shows the changing dimensions of the buccal periodontal space in relation to jaw movement and electromyography of jaw muscles. As the jaw closes (upward deflection of the bottom channel), the periodontal space narrows abruptly (downward deflection of the top channel), remaining narrow until opening begins. The horizontal bar represents 200 msec. The vertical bars represent 10 μ m for the root–buccal bone distance, 200 mV for the temporalis and digastric muscles, and 10° for jaw open–close. (Figure is courtesy Dr. Z.J. Liu, University of Washington.)

Loads on teeth during function

In vivo forces on teeth are not all vertical. The tongue loads the teeth horizontally from the lingual side during chewing and swallowing and is balanced by labial forces from the lips and cheeks (Thüer *et al.* 1999). Notably, tongue and cheek pressures can act on erupting teeth even before they come into occlusion. Anterior positioning of the tongue during swallowing (“tongue thrust”) is thought to be a cause of open-bite malocclusions (Kawamura *et al.* 2003). Orthodontic appliances such as tongue cribs and buccal shields are intended to change this balance and allow the teeth to move toward areas of lowered pressure (McDougall *et al.* 1982).

Forces from the powerful and anatomically complicated masticatory muscles are predominantly (but not

exclusively) vertical and are delivered to the occlusal surface of the teeth. Maximal voluntary bite forces are easily measured with transducers placed between the teeth. Depending on muscle morphology, age, gender, and probably sensory feedback and motivation, average maximal voluntary bite forces for molars range from about 150 N (female children) to over 400 N (male teenagers) (Palinkas *et al.* 2010). Force levels are lower at incisors (Roldan *et al.* 2009), but even here they reach levels sufficient to drive dentinal fluid into the pulp (Paphangkorakit & Osborn 2000).

Although static bite force is easy to measure, the dynamic occlusal forces of natural mastication are not. The approach is to instrument a crown or implant with force transducers. A pioneering study by Anderson simultaneously documented tooth-to-tooth contacts (Anderson 1955). More current studies use devices that can measure force in three dimensions. They have shown (1) mandibular molar vertical forces up to 450 N (i.e., at least as high as maximum voluntary bite force), (2) mesial and medial forces often exceeding 50 N, and (3) enormous variability among subjects, partially due to variation in chewing stroke (Mericske-Stern *et al.* 1992; Morneburg & Pröschel 2002).

Because of the difficulty of these studies and the fact that they cannot be undertaken with an intact dentition, there is great interest in numerical methods. Finite element modeling is suitable for exploring static biting under different periodontal conditions (Ona & Wakabayashi 2006; Qian *et al.* 2009). However, extrapolation to mastication requires dynamic methodology. Several promising approaches are in developmental stages (Maki *et al.* 2003; Rohrle *et al.* 2009; Hannam *et al.* 2010).

Ligament or fluid support?

At this point, we can address one of the most persistent questions in periodontal mechanics. When occlusion presses the tooth into its socket, what supports it—the periodontal ligament, hydrodynamic properties of the ground substance, or fluid pressure from the vascular compartment? Classically, the hammock-like arrangement of the principal fibers of the periodontal ligament was taken as *prima facie* evidence that the ligament, tensed by occlusal force, supported the root. This intuitively satisfying notion was challenged by work in the 1960s on rat incisors showing that, when tested after death, tooth mobility was greater and recovery from intrusion was very slow compared to in vivo readings (Bien 1966). The suggested mechanism was that fluid moved into the bone marrow during loading but could not return in the absence of a blood pressure gradient. This proposed mechanism was extrapolated to suggest that, during mastication, a constantly replenished fluid—

film layer was trapped between the ligament fibers and served to cushion the repeated loads (Bien 1966). The notion of fluid support was enhanced by the water-binding properties of the GAGs, which should enable the ground substance itself to resist compression (Embery *et al.* 1995). However, it is also obvious that ligament fibers are indispensable for normal mechanical behavior (Watanabe & Komatsu 1997), although the bound GAGs increase their strength (Kawada & Komatsu 2000). The most reasonable conclusion is that the periodontal tissues can resist both tension (via collagen) and compression (via GAGs). Indeed, because occlusal forces are never strictly intrusive, tipping and rotation inevitably cause areas of compression as well as tension. The remaining question is whether this occurs during normal function, or whether functional loads are sufficiently low that they do not engage the late phase of periodontal stiffness (cf. Table 30.1). While this seems possible for activities such as swallowing and speech, masticatory forces are typically large, and it is generally assumed that the periodontal ligament is tensed during chewing.

Overview

There are holes in our knowledge about functional aspects of the tooth–bone interface, both anatomical (root and alveolus in three dimensions) and physiological (ligament and fluid dynamics). Tooth mobility has been measured only under static loads, but because occlusal loading during mastication reaches levels as high as those during maximum voluntary biting, teeth probably move significantly with every chewing stroke. Tooth support during mastication most likely tenses the ligament fibers in some areas, while compression in other areas is resisted hydrodynamically.

However, for sustained loads (e.g., orthodontics), neither ligamentous nor fluid support is maintained, and focal necroses as well as inflammation occur in the PDL. In this instance, adaptation is accomplished by remodeling of the tissues instead, the topic of the “Modeling and Remodeling” section.

Modeling and remodeling in response to mechanical strain

Soft tissues

Mesenchymal tissues readily respond to mechanical loading, and the tooth–bone interface is no exception. Studies on periodontal ligament cells *in vitro* demonstrate that tensile force causes alignment (Bellows *et al.* 1982) and promotes osteogenic differentiation (Kawarizadeh *et al.* 2005; Kanzaki *et al.* 2006), whereas compressive force promotes osteoclastic inducers (Li *et al.* 2011). *In vivo* findings are similar in that the mag-

nitude of occlusal loading (which is presumed tensile) is correlated with the size, density, and mineralization of Sharpey’s fibers in rat periodontium (Short & Johnson 1990; Silva & Merzel 2004), whereas the periodontal ligament attachments are reduced in compressed locations (Fukui 1993; Zhao *et al.* 2008).

The most mechanically significant event in the life of a tooth is probably the moment when it comes into occlusion and begins to bear load. The stiffness of the periodontal attachment apparatus increases dramatically when erupting teeth reach occlusion (Popowics *et al.* 2009). That this is a causal relationship is shown by the fact that the stiffness remains low when the opposing tooth has been removed (Yeh *et al.* 2010).

The alveolar process: modeling

The alveolar process exists to house the dental roots and does not develop in their absence. Like all bone tissue, the alveolar process can respond to mechanical loads by alterations in physical size or shape (bone modeling) or by replacing old structure with new (bone remodeling). Modeling, which can be resorptive, appositional, or both at different surfaces, characterizes growth, eruption, drift, and sites of tension in orthodontic tooth movement. Remodeling, which is always ongoing, does not change outer form, but bone density and hence strength are modified by the balance between resorption and subsequent apposition. The periodontal space, like cranial sutures and the periosteum, possesses many cells with osteogenic potential. These tissues also are characterized by a rich vasculature, which means that cells with osteoclastic capacity can be recruited rapidly and may even be resident (Saffar *et al.* 1997; Ochareon & Herring 2011). Thus, the potential for both apposition and resorption is always present, along with an exquisite sensitivity to load. Coupled with the high turnover rate of alveolar bone, it is not surprising that the alveolar socket adapts readily to dental loading and that dentoalveolar changes generally contribute more to orthodontic correction than do other parts of the craniofacial skeleton.

Modeling of the alveolus is mechanically disruptive for the tooth–root interface. Tooth movement requires resorption of part of the alveolar socket and thus loss of attachment for the periodontal ligament. If this were to happen *en masse*, calamitous collapse might occur, but under natural circumstances, the process occurs piecemeal with minimal mechanical effects (Saffar *et al.* 1997).

In turn, mechanical loading causes some, but not all, alveolar modeling. One exception is pre-emergent eruption, which is independent of mechanics (Wise & King 2008) and precedes the development of the tooth–bone interface as discussed in this chapter. Mechanics are important for post-emergent eruption, especially after

occlusion is achieved; for these later stages, blood pressure promotes (Shimada *et al.* 2006) and occlusal loading retards or reverses (Kuster & Ingervall 1992) extrusive movement. Most evidence indicates that the mechanical influence that operates on eruption is pressure, not traction from the ligament (Moxham & Berkovitz 1995). Previous work, however, has concentrated on the position of the erupting tooth, and little is known about why alveolar bone so readily accompanies the root in its migration toward the oral cavity. Lightening the occlusal load increases alveolar height, but this seems to be secondary to overeruption of teeth (Kingsmill *et al.* 2010; Yeh *et al.* 2010; Yeh & Popowicz 2011). It is conceivable that tensile forces from crestal periodontal ligament fibers play a role in adding bone to the crest, but this idea, which could also explain why the apex of the socket is also appositional (Wise & King 2008), has yet to be tested.

Drift is a form of modeling that continues through life; although teeth drift mesially in most mammals, including primates and pigs (Yilmaz *et al.* 1981), drift is distal in rats and mice. The involvement of mechanical loading has been debated for decades and is summarized elsewhere (Moxham & Berkovitz 1995; Luan & Diekwisch 2007). These early studies showed that occlusal force might influence drift but was not essential. In contrast, the most likely mechanical component was traction from transeptal collagen. If so, then the pull of these fibers suffices to provide an impetus for directional modeling of the alveoli that resembles orthodontic tooth movement.

Orthodontic tooth movement is the unquestioned epitome of mechanically induced modeling of alveolar bone, because it occurs in direct response to loads applied to teeth. Furthermore, both animal experiments and clinical experience consistently show bone resorption in areas where the root comes into closer proximity to the socket ("pressure side") and bone apposition in areas where the root moves away from the bone, presumably putting traction on the ligament ("tension side"). Both regions are characterized by increased vascularity and collagen turnover (Rygh *et al.* 1986) and thus may represent some level of injury. The osteogenesis on the tension side corresponds with observations of oriented, mineralizing ligament fibers (Rygh 1976). The greatly elevated osteoclastic activity on the pressure side is usually ascribed to mechanical damage that triggers bone remodeling, specifically microcracks (Verna *et al.* 2004) and hyalinized, avascular areas of the periodontal ligament (Rygh *et al.* 1986). Some numerical simulations have suggested that rather than being caused by compressive stress, resorption follows loss of loading (i.e., disuse atrophy) (Melsen 1999; Cattaneo *et al.* 2005).

However, this computational prediction is contradicted by empirical evidence of traumatic loading (Verna *et al.* 2004). The traditional compression–resorption, tension–apposition dichotomy is still the best available explanation for alveolar modeling during orthodontic tooth movement.

The alveolar process: remodeling

Remodeling of bone is a feature of mammalian physiological homeostasis, but it also is a means to repair mechanical damage and to adapt structure to mechanical needs by increasing or decreasing mineral density. As with bone generally, hypofunction, such as occurs with a soft diet, leads to osteoporotic changes, whereas higher occlusal loading promotes bone density (Thongudomporn *et al.* 2009). These adaptations can occur to some degree even in adult animals (Mavropoulos *et al.* 2010).

As mentioned, remodeling of alveolar bone is rapid even in adults, and because the bone is relatively young, its mineralization and hence stiffness are low. This raises the question of cause versus effect. Is alveolar bone flexible because it is adapted to avoid stress concentration (Daegling *et al.* 2011) and to assist the periodontal soft tissues in their deformation, or is the flexibility a byproduct of the rapid turnover (itself a response to damage or the need for tooth movement) that actually weakens the system? As yet, we have too little information on in vivo mechanics to approach an answer.

The craniofacial skeleton as a whole

The alveolar processes are part of the maxilla and mandible, which in turn are elements of the entire skull. Mechanical loading of the tooth–bone interface loads all the elements directly or through sutures and joints. During function, loading also comes from muscle contraction, and these forces place their own loads on the mandible and cranium. Animal studies indicate that the occlusal force itself, rather than any individual muscle, is primarily responsible for shearing the maxilla and mandible in opposite directions, while the temporomandibular joint and braincase loads depend on which muscle is most active (Herring *et al.* 2001). Because of the complex choreography of muscle contraction during mastication, the strains experienced by the skull are extremely dynamic, sometimes changing from compression to tension and back to compression within the time of a single power stroke (Figure 30.4). Evidence that muscle and occlusal forces are important for modeling and remodeling the entire skull comes from (1) human studies showing an association of muscle and occlusal force with a short-faced growth pattern (Proffit & Fields 1983; van Spronsen *et al.* 1997), and (2) dietary manipu-

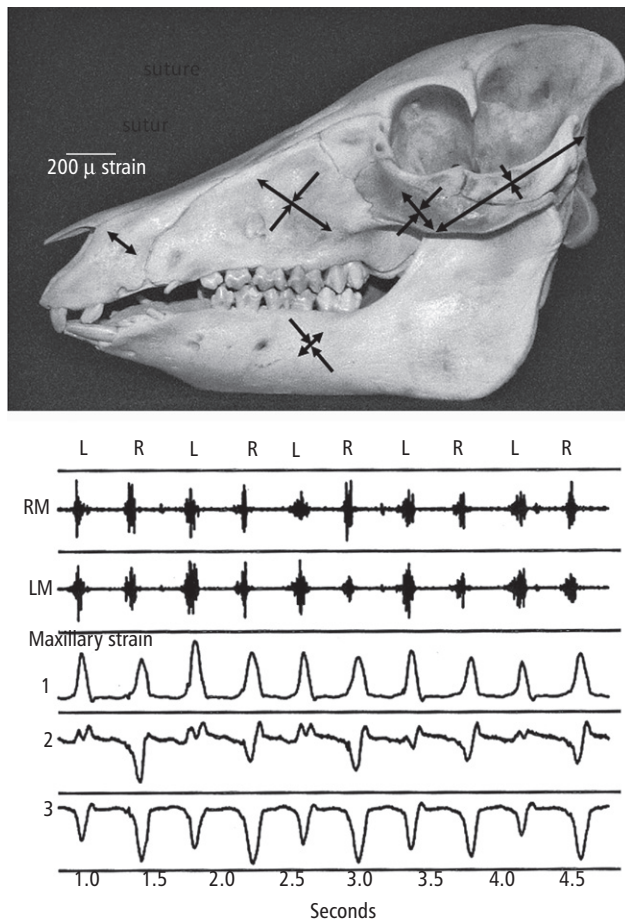


Figure 30.4 The upper figure shows average bone strain on the working side during *in vivo* mastication in pigs. Tensile strain is indicated by lines with divergent arrowheads, and compressive strain by lines with convergent arrowheads. Note that the maxilla and the mandible show opposite patterns. In the maxilla, compressive strain is oriented backward and upward from the alveolar bone to the cranium, whereas in the mandible compressive strain is oriented backward and downward toward the inferior border. The recording shows strain from a three-channel gage on the left maxilla in relation to muscle activity in the right (RM) and left (LM) masseters. The pig alternates between right (R) and left (L) chewing cycles. In every cycle, channel 1 (horizontal orientation) shows tensile strain (upward deflection) and channel 3 (vertical orientation) shows compression (downward deflection). However, channel 2 (45°) is highly dynamic, typically showing two peaks per cycle, both tensile for left cycles and compression followed by tension for right cycles (Herring *et al.* 2001).

lation in animals showing that low loading is associated with reductions of transverse growth and of bone density as far away from the jaws as the sagittal suture (Burn *et al.* 2010).

Orthodontic forces on teeth also have effects on the entire skull. Appliances such as palatal expanders, headgear, and reverse headgear are typically attached to teeth,

but they are intended to act on craniofacial sutures as well. Measurements on dry skulls and numerical simulations also indicate that such appliances are likely to cause strain at distant locations such as the cranial base, the zygomatic arch, and sutures around the frontal bone, but there is debate about whether the magnitude of such strain is sufficient to cause bone adaptation (Miyasaka-Hiraga *et al.* 1994; Oberheim & Mao 2002; Holberg *et al.* 2007). To date, none of the models have been validated.

Overview

Tensile strains in the periodontal space promote ligament alignment and produce osteogenesis, whereas compressive strains are associated with loss of ligament attachment and resorption of bone. Understanding the location and orientation of these strains helps to explain some aspects of post-emergent eruption and drift and is the basis of orthodontic tooth movement. The magnitude and pattern of habitual loading are influences on density and growth not only of the alveolar bone, but also of the entire craniofacial skeleton.

Alveolar bone mechanics without teeth

Biomechanical consequences of tooth absence or loss

It is a truism that alveolar bone does not exist without teeth. If teeth are lost, alveolar bone is quickly resorbed. One of the most interesting questions in dentistry is why the socket dissolves so rapidly when the root is removed. As reviewed by others, loss of oral bone is more correlated with loss of function than with age or osteoporosis (Kingsmill 1999; Boskey & Coleman 2010). The critical factor presumably is mechanical strain (Fleischmannova *et al.* 2010), but whether the strain is decreased or elevated is not clear. Intuitively, in the absence of teeth, loading and hence strain would be reduced. Edentulous subjects have weaker muscles and lower occlusal force than age-matched controls (Caloss *et al.* 2010), which supports the idea of decreased strain. Bone loss could then be attributed to disuse atrophy changing the remodeling balance to resorptive, the rapidity of the process being simply due to the intrinsically fast turnover of alveolar bone. Areas of greatest bone loss do seem to be those with the highest turnover rates (Kingsmill 1999). Such a scenario is appealing, but it is in conflict with the notion that fast turnover is itself a response to mechanical damage. Alternatively, it is possible that tooth loss increases or redistributes bone strain. As structures, jawbones are strikingly different from other bones by the very fact that they have large defects—the alveoli. When occupied by roots and healthy soft tissue, these structural

defects confer no mechanical disadvantage, but extensive periodontal disease or removal of teeth increase strain by making the mandible, as a whole, less stiff (Daegling *et al.* 1992). Apposition and stiffening of some regions of cortical bone occur in the edentulous mandible, a finding that suggests that limited areas may be under increased loading even if overall levels are decreased (Kingsmill 1999; Schwartz-Dabney & Dechow 2002).

Implants

Because osseointegrated implants lack the periodontal space and soft tissue that allow teeth to adjust their position, their bone–tooth interface is much less flexible and presumably has stiffness more closely resembling alveolar bone than periodontal ligament. Functionally, however, the difference may not be as large as the figures compiled in Table 30.1 would suggest, because the dynamic loading of chewing probably causes viscoelastic stiffening of the natural ligament (Sanctuary *et al.* 2005). Although less responsive than dentate subjects to food consistency (Grigoriadis *et al.* 2010), subjects with implants have normal magnitudes of occlusal loading. However, these normal loads are thought to cause higher stresses in the bone because of the absence of the stress-absorbing soft tissues (Ren *et al.* 2010). In addition, the directionality of the loads conveyed to bone by the implant is different from that conveyed by the stretched periodontal ligament. While some bone loss occurs after implant placement, it is minimal compared to the bone loss that occurs with no treatment or dentures, and the best preserved sites seem to be those under the greatest stress (von Wowern & Gotfredsen 2001). Thus, functional loading of the alveolar bone may be the critical factor in its preservation. Nevertheless, questions remain about how removal of the periodontal space itself affects alveolar remodeling. Elimination of this space subtracts a major source of osteoprogenitors, and the absence of periodontal circulation may limit access for osteoclast precursor cells. Therefore, one might expect bone around implants to be older, more mineralized, and stiffer than bone around natural teeth, and the better preservation might simply reflect lower turnover.

Overview

Edentulousness is associated with decreased overall loading that, coupled with high turnover, is the most likely cause of alveolar bone loss. However, the changed pattern of loading probably raises strain levels in some locations, accounting for site-specific stiffening. The better maintenance of bone around implants may be the result of loading levels that are more normal or lower turnover.

Implications for future translational research on PDL biomechanics

The tooth–bone interface serves one of the most important behaviors in mammals—chewing. Its short-term properties subserve the mechanical needs of breaking down foods, while its long-term adaptability enables adjustments to changing dental conditions and is the basis of orthodontic treatment. The mechanical behavior of the periodontal soft tissues is a key element in the ability of the interface both to protect the dentition and to maintain the alveolar bone. However, the mechanics of the tooth–bone interface are complex, and each patient presents unique features related to occlusal pattern and jaw muscle usage. Although there is now a rich literature on the material properties of periodontal constituents, we are lacking information about how the interface behaves during function. Animal studies are probably the best way to obtain missing information such as the nature of tooth mobility during chewing. While numerical simulations will eventually be the way to attack these problems in humans, computational modeling is still in an early stage, and too many current examples are not validated. Ultimately, however, customized treatment can be envisioned in which dynamic modeling of functional occlusion could be carried out for each patient.

Summary

The mechanical behavior of teeth depends both on properties of the constituent materials and on the nature of functional loading. Unique elements of the periodontal apparatus include the ability of the ligament to resist both tension and pressure, specializations at the tissue boundaries, and a high turnover rate for bone as well as soft tissue, all of which are mechanically related. Fluid pressure and blood flow are important elements, necessitating *in vivo* studies for a full understanding. The periodontal apparatus is relatively rigid and elastic for short-term loads like mastication, but pliable and plastic for long-term loads like orthodontic treatment. Functional loading of the tooth–bone interface is essential for maintenance of alveolar bone and influences the size and shape of the entire skull. Ultimately, the complex mechanics of the tooth–bone interface will be understood using numerical simulations unique to each patient.

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Clinical correlate: biomechanics of teeth in bone

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For several decades the demographics of patients seeking restorative dental treatment have been changing toward increasing numbers of adults. In a survey covering the period from 1980 to 1995, dentists' income per patient for the younger age groups trended downward for restorative dental services and upward for preventive procedures. However, the restorative types of treatments remained high for middle-aged and older adults (Eklund *et al.* 1998). These trends are thought to be the result of successful caries prevention approaches for children, along with the aging of the "baby boom" generation. The fact that these trends have continued and have resulted in increased demands for orthodontic services by adults is further supported by the periodic practice surveys done by the *Journal of Clinical Orthodontics*. From 1993 to 2009, the annual number of active adult patients per orthodontist rose by 34.8% and the number of new adult case starts increased by 37.5% (Keim *et al.* 2009).

Adults can present the orthodontist with multiple challenges that are not common in adolescents. These include reduced tooth support from previous periodontal disease, partial edentulism with accompanying ridge atrophy and tooth drift, dental attrition with irregular gingival margins, and complex restorative needs. These problems often are compounded by superimposed malocclusions, including dental misalignment and skeletal jaw discrepancies. Several new approaches are now available that can impact alveolar bone turnover and tooth movement control while addressing some of the challenges presented by the adult orthodontic patient.

Orthodontic treatment is not contraindicated for adults with reduced, but otherwise healthy, periodontium (Artun & Urbye 1988; Boyd *et al.* 1989; Re *et al.*

2000). However, since the centers of resistance are displaced apically, conventional appliances tend to generate greater moments and more extrusion, requiring alterations in traditional biomechanics to reduce forces while controlling tipping (Jeon *et al.* 2001). New superelastic metal alloys and wire configurations are now available, and these can deliver fairly constant forces with low magnitudes while simultaneously controlling moments (Rose *et al.* 2009). Frequently, adults who have experienced loss of periodontal support also have reduced sources of anchorage for required tooth movements. Today, this deficiency can be addressed simply with the use of temporary anchorage devices (TADs), which are easily placed and provide excellent anchorage sites to support complex tooth movements, many of which could not easily be achieved using conventional tooth-borne anchorage (e.g., molar intrusion; Leung *et al.* 2008). These approaches also can be combined with accelerated orthodontic tooth movement to substantially reduce treatment times, while enhancing desired movements (Kim *et al.* 2009; Wilcko *et al.* 2009). Accelerated orthodontic tooth movement relies on stimulation of localized alveolar bone remodeling in response to localized injury to enhance tooth movement.

Adults also have an increased prevalence of edentulous spaces with accompanying tooth migrations. Characteristically, dental migration is marked by mesial tipping and rotation of molars, dental spacing with unfavorable tooth positions, and supereruption of unopposed teeth. Restoring adult patients to optimal oral health, function, and esthetics often requires a course of orthodontics to correct tooth migrations and prepare the case for optimal restorations. These preparative

tooth movements can have added benefits such as improvement of unfavorable crown-to-root ratios, reduction of infrabony defects, improvement of occlusal relationships, and establishment of conditions for more conservative restorations (Brown 1973). Movement of teeth into adjacent bony defects has been shown to improve bone fill, but it does not provide significant gains in attachment (Cirelli *et al.* 2003; Corrente *et al.* 2003). However, molar uprighting with an extrusive component at the defect site can result in a more favorable position of the connective tissue attachment along with defect reduction and, if the crown is reduced, improved crown-to-root ratio (Iino *et al.* 2008).

Dental attrition is another common condition seen in adults, resulting in uneven gingival margins and poor esthetics. Adults often seek treatment for these elective esthetic problems. Treatment should involve examination of the depth of the dentogingival sulcus and a determination of whether gingivectomy or orthodontic treatment would be the more appropriate approach. Dental extrusion and intrusion will correct gingival margins, but it often results in incisal edge irregularities, which need further treatment with either odontoplasties or restorations to achieve optimal dental esthetics (Kokich 1993, 1996).

Adults who grew up before widespread fluoridation of municipal water supplies characteristically present with inadequate dental restorations and other restorative needs. Prior to removal of nonrestorable teeth, it is often wise to consider dental extrusion as an effective means to prepare the alveolar ridge for implant placement. The osteogenesis that accompanies slow orthodontic extrusion is a viable alternative to surgical bone augmentation procedures that may otherwise be required to prepare an implant site following extraction of nonrestorable teeth (Korayem *et al.* 2008). Movement of teeth into edentulous spaces, with or without subsequent extraction, also can improve ridge height and width because of the osteogenic response of alveolar bone to tensile strains in the periodontal ligament (Lindskog-Stokland *et al.* 2011).

The overriding goal for any adult orthodontic treatment should be to balance esthetic and functional improvement with cost and time in treatment. Because adults often require input from multiple dental disciplines, their treatments often result in high cost and extended treatment times. Therefore, planning and coordinating treatment are of paramount importance to achieving successful outcomes with maximum efficiency (Spear *et al.* 2006). Possibilities for compromise that reduce costs and treatment time always should be considered. To illustrate, the ideal treatment for many crowded dentitions often involves the extraction of per-

manent teeth. Most orthodontists feel that, when indicated, dental extractions are a better choice than flaring incisors because the latter may be accompanied by greater risk of gingival recession and less stability. However, aligning incisors by flaring in adults can lessen time in treatment and be an acceptable compromise, although this may require some form of permanent retention as well as careful assessment of esthetics and the risk of gingival recession. The risk of recession following dental advancement in adults is influenced by the presence of recession at baseline, the gingival biotype, the width of keratinized gingiva, and the presence of gingival inflammation (Melsen & Allais 2005).

Case presentation

Our patient serves as an excellent example of how newer biomechanical approaches can be employed to manage adults with complex restorative needs. He is a 33-year-old Caucasian male dentist with chief complaints of poor dental esthetics and function due to the loss of his maxillary right canine and lateral incisor, accompanied by severe loss of the edentulous alveolar ridge (Figure 31.1), gingival recession, and an unsatisfactory prosthetic replacement (Figure 31.2A). The endodontically treated maxillary right first premolar also was characterized by a periapical lesion and severe mesial bone loss, but there was adequate height of the connective tissue attachment on the distal side (Figure 31.1A). The patient's medical history was noncontributory. Although he did undergo orthodontic treatment at age 18 to take care of the impacted maxillary right canine, the treatment was unsuccessful and resulted in severe root resorption of the maxillary lateral incisor due to impingement of the canine crown against the root of the lateral incisor. The canine and lateral incisor ultimately were extracted, an unsuccessful bone graft in the edentulous space was attempted, and a five-unit bridge was eventually placed. The bridge consisted of an anterior abutment on the maxillary right central incisor and posterior abutments on both maxillary right premolars. Pontics replaced the maxillary right lateral incisor and canine (Figure 31.2).

Since the patient desired implants to replace the missing teeth and an initial attempt to graft the atrophic edentulous ridge failed, the goal of his orthodontic treatment was to develop the edentulous site for implants. The maxillary right first premolar was considered to be nonrestorable, but it would be useful as a means to develop alveolar bone in the edentulous sites for subsequent implant placement because the bone height on the distal was adequate. The plan was to detach the first premolar from the bridge and move it mesially through

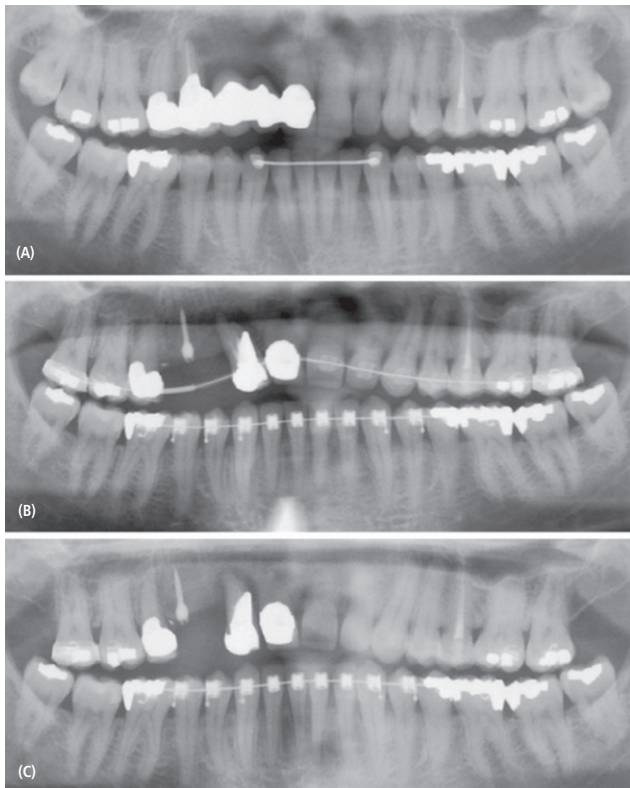


Figure 31.1 Panoramic radiographs. A. Pretreatment. B. Progress with temporary anchorage device (TAD). C. Implant site preparation completed prior to extrusion and extraction of maxillary right first premolar and implant placement.

the atrophic ridge to stimulate osteogenesis by placing the distal periodontal ligament under tension. Temporary anchorage devices would be used, as needed, to conserve anchorage in the posterior part of the maxillary right quadrant while accelerated tooth movement techniques would be used to facilitate tooth movement in the anterior segment of the dental arch aimed at correcting the existing end-on incisor relationship.

A full edgewise orthodontic appliance was placed, and the pontics were bracketed and tied to the archwires. The pontics were removed gradually as the first premolar moved mesially. This approach provided the patient with maximum esthetics during treatment. Both dental arches were leveled, aligned, and coordinated. After sectioning the connection between the maxillary right premolars, the maxillary first premolar was mesialized using open coil springs on the archwire between the premolars. This continued for five months with an excellent osteogenic response distal to the tooth (Figure 31.1B). Once sufficient bone was generated distal to the first premolar, a miniscrew temporary anchorage device was placed in the site to provide additional anchorage for the posterior

teeth in the maxillary right quadrant (Figures 31.1B–C and 31.2C). When the first premolar abutted the right central incisor (Figures 31.1C and 31.2 C–F), extrusion and extraction of the first premolar and immediate implant placement were done (Figure 31.3). The ultimate restorative plan (Figure 31.4) was to place three single tooth crowns on the implants to replace the maxillary right lateral incisor, canine, and first premolar.

Discussion

The osteogenic response to tensile strains on the periodontal ligament is well known and has a long history of use by orthodontists to treat malocclusions. With the emergence of implants to restore missing teeth, clinicians began to exploit this osteogenic response to tension to generate new alveolar bone for implant sites. Although orthodontists are familiar with this approach to stimulating osteogenesis for this particular clinical outcome, the mechanisms underlying it remain unclear. Briefly, four interrelated steps are involved: (1) sensing the mechanical signal by the periodontal ligament cells, (2) transduction of mechanical into biochemical signals, (3) transmission of biochemical signals into the cell nuclei, and (4) synthesis of new molecules by the effector cells (Wise & King 2008). Oscillating fluid flow in the confined space of the osteocyte lacunar-canalicular system may be an important mechanosensing signal in cortical bone (You *et al.* 2000). However, the primary responders in the PDL appear to be mesenchymal cells, and these may be affected more by strains in the extracellular matrix in their microenvironment (Chan *et al.* 2010). The transduction of mechanical into biochemical signals is accomplished via the integrin–actin cytoskeletal mechanism, alterations in membrane channels, and activation of several receptors that reside in the cell membrane. Several intracellular signaling pathways are then activated to transmit this information to the cell nuclei. The latter control the synthesis of important new effector molecules that are either secreted (e.g., matrix proteins or growth factors) or used by the cells (e.g., microtubules; Hughes-Fulford 2004).

Recently, TADs have revolutionized orthodontic biomechanics. Tooth ankylosis and implants serve to demonstrate the essential role of the periodontal ligament in tooth movement. Ankylosed teeth have focal lesions characterized by bony bridges that eliminate the ligament in these areas. Similarly, implants, with or without osseointegration, also lack a periodontal ligament. In both cases, teeth are unresponsive to orthodontic tooth movement and dental drift. An appreciation of this biological background has provided the impetus for the recent wide acceptance by orthodontists of TADs, which



Figure 31.2 Intraoral photographs. A and D. Pretreatment. B and E. Treatment progress. C and F. Pre-extraction and implant placement.

do not move because they lack a periodontal ligament. Miniscrews have been particularly popular because they can be placed and removed easily by the clinician and provide excellent stable anchorage to support complex tooth movements.

Periodontal accelerated osteogenic orthodontics (PAOO) is another biological approach that combines a minor surgical alveolar trauma—usually corticotomy—with orthodontics to increase alveolar bone width, shorten treatment time, and increase post-treatment stability. The theoretical basis for the approach rests on the bone-healing pattern known as the *regional acceleratory phenomenon* (RAP). The RAP manifests as an increase in bone remodeling surrounding sites of bone injury (Wilcko *et al.* 2009).

PAOO is not as widely used as TADs and tensile osteogenesis, probably because it is more invasive and rests

largely on an evidence foundation of case reports. The promise of accelerated orthodontic treatment with greater efficiency and safety remains to be fully realized, but whether this can best be done surgically or pharmacologically remains an open question today.

Summary

There is an increased demand for orthodontic services by adults. Often these patients require complex and multidisciplinary treatments. Approaches to treating these problems that rely on better control of alveolar bone remodeling have recently become available. This chapter reviews some of these approaches and presents a case that relies on their use to achieve an acceptable outcome. The patient presented with missing maxillary right lateral incisor and canine teeth, an atrophic edentulous

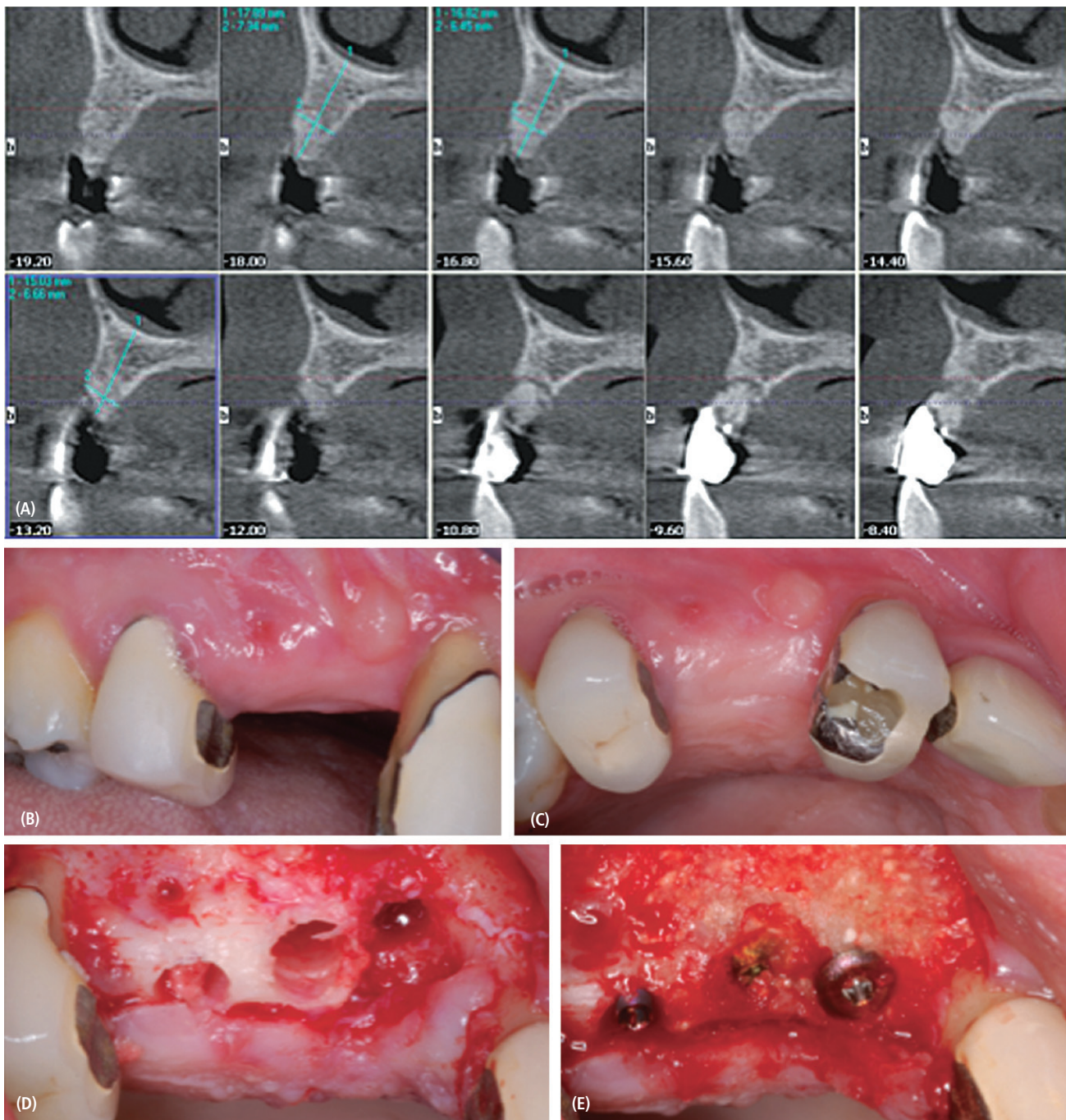


Figure 31.3 Surgical photographs and radiographs. A. CBCT slices of the implant site showing bone heights and widths (note in panel A that maximum alveolar bone height = 17.9 mm and width = 7.3 mm). B. Lateral view of the surgical site. C. Occlusal view of the surgical site. D. Post-extraction and implant sites. E. Implant placement (all implants were 13 mm long).

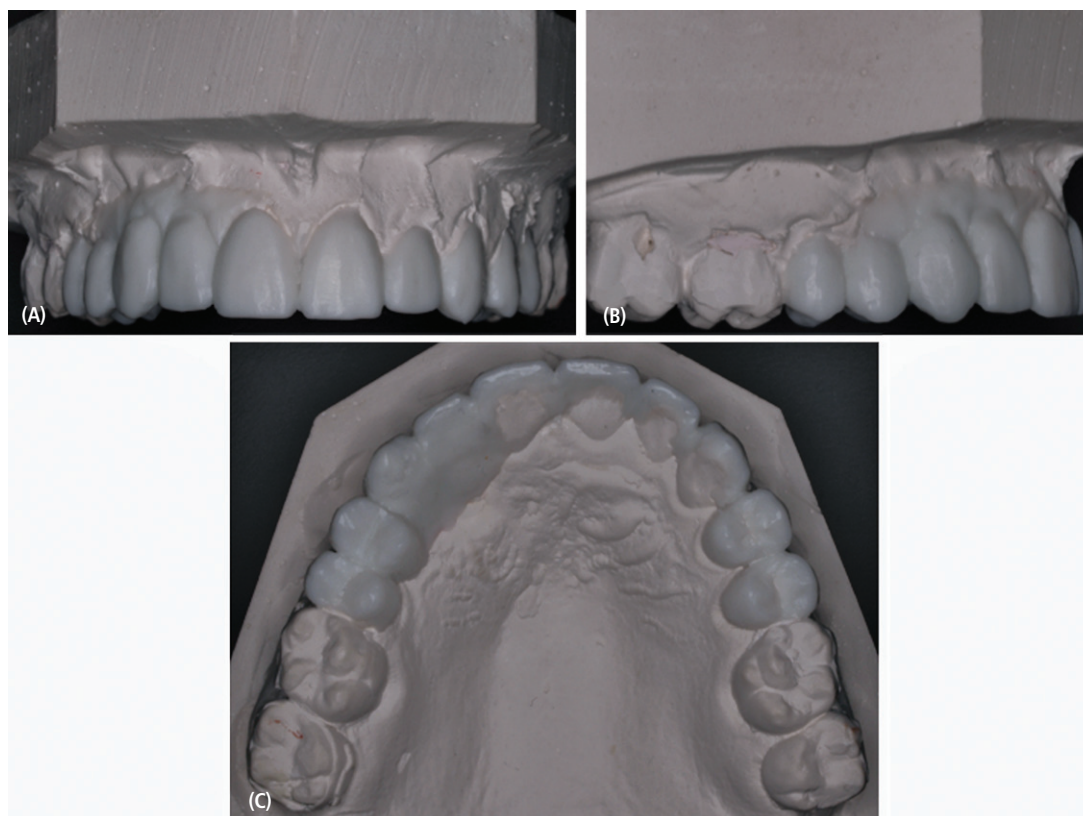


Figure 31.4 Restorative diagnostic setup. A. Frontal. B. Implant site. C. Occlusal.

ridge, and severe bone loss on the adjacent maxillary first premolar. The main goal of treatment was to develop the edentulous site for placement of implants. The adjunctive approaches used were tensile osteogenesis, a temporary anchorage device, and accelerated orthodontics. Alveolar bone height was increased in the edentulous ridge and was considered to be acceptable for the placement of implants and ultimate prosthetic replacement of the missing teeth.

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Impact of metabolic bone disease on craniofacial bones and teeth

Jill Bashutski, L. Susan Taichman, and Laurie K. McCauley

Metabolic bone disease is a broad term used to describe conditions of altered bone metabolism. Osteoporosis, hyperparathyroidism, renal osteodystrophy, and Paget's disease are the most prevalent of these diseases. The most common form of osteoporosis is postmenopausal, although other categories, including age-associated, glucocorticoid-induced, and cancer-related osteoporosis, are also important. A defining feature of osteoporosis is its multifactorial etiology.

Periodontitis is a widespread condition that is a significant cause of tooth loss in society, especially in the elderly. Data from the third National Health and Nutrition Examination Survey (NHANES) indicate that up to 80% of American adults have some degree of periodontal disease (Dye *et al.* 2007). The prevalence of complete edentulism in adults aged 65–74 years is estimated at 24%, much of which may be attributed to periodontal disease. Periodontal disease can negatively impact overall quality of life due to impaired speech, esthetics, and function as a result of gingival recession, infection, tooth mobility, and tooth loss. More importantly, an association between periodontal disease and other systemic manifestations of disease has been recently established. Untreated periodontal disease may contribute to increased preterm low-birth-weight births, cardiovascular disease, diabetes, and other systemic conditions (Scannapieco 2005).

Since periodontitis is a bone-resorptive disease, there is biologic rationale to suggest that alterations in systemic bone metabolism may influence oral bone disease progression. Moreover, therapies used to treat metabolic bone disease further alter bone metabolism, which also affects bones of the oral cavity. This chapter illustrates

the key features, epidemiology, and etiology of the most common metabolic bone diseases including osteoporosis, hyperparathyroidism, renal osteodystrophy, and Paget's disease. In addition, oral findings unique to these patient populations are described.

Postmenopausal osteoporosis

Postmenopausal osteoporosis is a systemic bone disease that is characterized by a period of relatively rapid loss of trabecular bone mineral density (BMD), as well as less pronounced loss of cortical BMD due to the decline in gonadal hormone secretion following menopause. These alterations in the macro- and micro-architecture of the bone lead to reduced bone strength and increased fracture risk, which is associated with a high degree of morbidity and mortality.

The World Health Organization (WHO) defines osteoporosis as a condition where the BMD of an individual is at least 2.5 standard deviations below the average bone density of a healthy 20- to 29-year-old-adult of the same gender (WHO 1994). A T-score is used to classify these standard deviations and correlates directly with the standard deviation. Thus, a diagnosis of osteoporosis is made when the T-score is lower than –2.5. A less severe form of systemic bone mineral density loss, osteopenia, is defined by a T-score between –1.0 and –2.5. A Z-score is less commonly reported and is based on the standard deviation from an age- and gender-matched healthy adult. Since not all patients who are classified as osteoporotic as defined by T-scores experience pathologic symptoms, osteoporosis may be defined by fracture incidence. In clinical trials for

osteoporosis therapeutics, outcome analyses rely heavily on fracture prevention versus BMD to demonstrate effectiveness.

Bone mineral density can be assessed using several methods, including dual-energy X-ray absorptiometry (DXA), multidetector computed tomography (MDCT), high-resolution peripheral quantitative imaging (HR-pQCT), and magnetic resonance imaging (MRI; Krug *et al.* 2010). DXA of the hip is the most common method used to assess systemic BMD; however, it has several limitations including the inability to account for different bone sizes or to differentiate between cortical and cancellous bone. HR-pQCT and MDCT provide the highest resolution imaging of trabecular bone structure, although imaging of central structures is not possible with HR-pQCT and MDCT requires high radiation doses. Recently, a tool for evaluating the risk of fracture in patients was developed by WHO (www.sheffield.ac.uk/FRAX/). This easy-to-use tool is based on data from large cohorts of patients that were used to develop algorithms to predict fracture risk with a 10-year probability. This tool can be used by healthcare professionals as well as patients.

Epidemiology

Osteoporosis is a critical public health problem with both economic and physical implications. It is estimated that nearly 12 million Americans over 50 years of age have osteoporosis and another 40 million have low bone mass, which puts them at risk for developing osteoporosis. The National Osteoporosis Foundation (NOF) expects that by 2020, estimates will increase to 14 million cases (NOF 2010). There are an estimated 2 million fragility fractures in the United States each year. With over 200 million individuals affected worldwide, osteoporosis is the most common metabolic bone disorder (Johnell & Kanis 2006).

The prevalence of osteoporosis has been estimated to be 55% in Americans over 50 years of age. Approximately 80% of those affected by osteoporosis are women (NOF 2010). Women have lower peak bone mass and smaller bone size, experience menopause (a period of accelerated bone loss), and have greater longevity when compared to men. Using the WHO T-score definition of 2.5 standard deviations below mean bone mass, 20% of Caucasian women, 20% of Asian women, 10% of Hispanic women, and 5% of African American women are estimated to have osteoporosis (NOF 2010).

Male osteoporosis is not as common compared with postmenopausal osteoporosis in women. For men over 50 years of age in the United States, 7% of Caucasian men, 7% of Asian men, 3% of Hispanic men, and 4% of African-American men are estimated to have osteoporosis. Although osteoporosis pathogenesis in men is not as

well understood, an age-related reduction in bone formation is thought to be the predominant mechanism (NOF 2010).

The presence of certain risk factors may significantly increase the risk for osteoporosis. These include family history, gender, age, ethnicity, previous or current use of systemic corticosteroids, anticonvulsant therapy, premature menopause, primary or secondary amenorrhea, primary or secondary hypogonadism in men, physical inactivity, smoking, excessive intake of alcohol, hormone replacement therapy (HRT), low dietary calcium intake, and vitamin D deficiency (NOF 2010).

The primary risk associated with osteoporosis is fracture. The most commonly noted fracture sites are typified by large amounts of trabecular bone such as the hip, spine, and wrist. The incidence of all fractures increases with age. As life expectancy increases worldwide and the number of elderly individuals rises, the incidence of fracture is anticipated to increase from 1.66 million in 1990 to 6.26 million in 2050 (Harvey *et al.* 2010).

The costs of osteoporosis and related fracture are substantial. Hip fracture is a major cause of hospital admission in the elderly. The acute care cost associated with hip fracture is tremendous in all developed countries. In 2005, the direct cost of hip fractures was around \$19 billion in the United States, and these costs are expected to increase to \$23.5 billion by the year 2025 (NOF 2010). By 2050, the worldwide cost burden of osteoporosis (for all ages) is estimated to increase to nearly \$131.5 billion (Harvey *et al.* 2010).

All fractures result in some degree of morbidity, but fractures of the vertebra and hip carry substantial mortality risks. On average, 24% of hip fracture patients over the age of 50 die during the first year after their hip fracture, with the highest mortality occurring in the first six months (NOF 2010). It has been estimated that there are around 740,000 deaths per year stemming from hip fracture, which represents 0.1% of the global burden of disease worldwide (Johnell & Kanis 2006).

Etiology

At the cellular level, osteoporosis represents a disruption in the normal balance of bone resorption (mediated by osteoclasts) and bone formation (mediated by osteoblasts) in favor of bone resorption. Cells of the osteoclast lineage (see Chapter 3 in this volume for a complete description of osteoclasts) are induced to differentiate by macrophage-colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL). Mature multinucleated osteoclasts have a half-life of 2–3 weeks in humans and are responsible for degrading the bone matrix. Osteoblastic precursors are induced to differentiate into mature matrix-producing osteoblasts (see

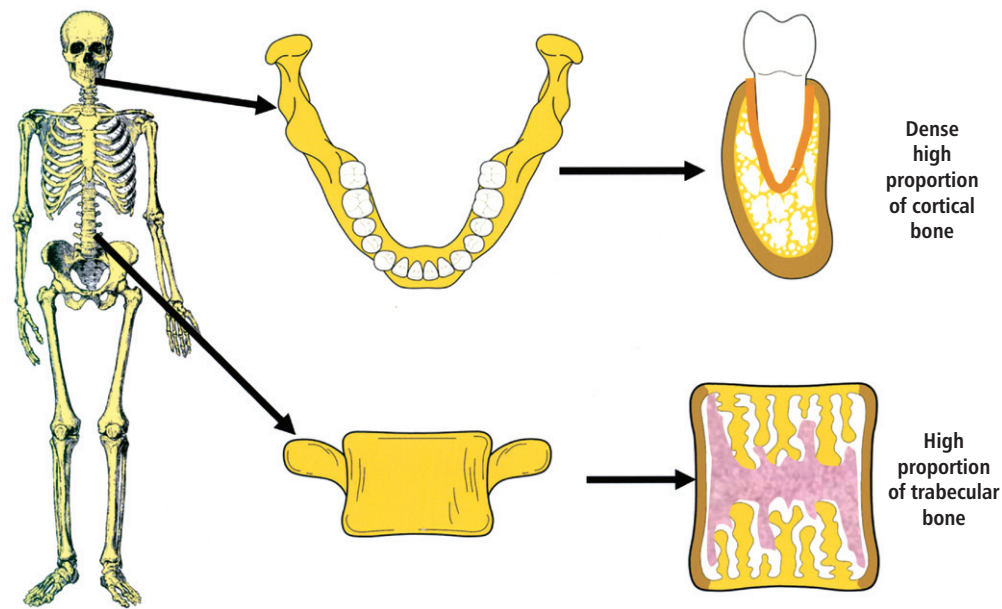


Figure 32.1 Differences in bones from various skeletal sites. The mandible is of neural crest origin and forms via intramembranous bone formation. At the organ level, the bone of the mandible is dense with a high proportion of cortical bone. The vertebral bodies have mesodermal origins and form via endochondral bone formation. Vertebrae have a high proportion of trabecular bone.

Chapter 6 in this volume for a complete description of osteoblasts) that lay down collagen and induce its mineralization to form the mature osseous matrix. Osteoblastic bone formation and osteoclastic bone resorption are coupled such that signals released during resorption are responsible for recruiting osteoblastic cells to replace the degraded bone. The process of bone formation to restore bone takes much longer than bone resorption, and hence, any increase in bone remodeling typically results in a net loss of bone. Although the processes of resorption and formation balance each other in net tissue gain or loss, the cellular requirements in a temporal sense are very different. The cellular impact at various skeletal sites is also different. Trabecular bone is more responsive to metabolic alterations and, therefore, is the target of hormonal flux, whereas cortical bone is less affected. This is an important point when considering how hormonal influences impact different skeletal sites. In states of normal bone balance where formation equals resorption, it is estimated that there are a million basic multicellular units (BMUs) active in the skeleton at any one time with a net turnover of 10% of the skeleton during a one-year period (Manolagas 2000). In theory, this would result in a totally new skeleton in humans every 10 years; however, it is not this simple since different skeletal sites turn over at different rates depending on their ratio of cortical to trabecular bone, biomechanics, and other influences. Because the mandible consists largely of dense cortical bone and in the dentate state

experiences regular biomechanical stimulation, it is less impacted by systemic hormones than the osteoporosis target bones such as the vertebrae and femoral bones (Figure 32.1).

Postmenopausal bone loss occurs in response to sex steroid withdrawal in concert with the cessation of normal menstruation. Estrogen via the estrogen receptor alpha ($ER\alpha$) increases osteoclast apoptosis and reduces osteoblast and osteocyte apoptosis (see Chapter 8 in this volume for a complete description of osteocyte biology; Figure 32.2). Estrogen also increases the production of IGF-1 and TGF β , both of which have positive effects on bone formation. The estrogen deficiency that occurs at menopause results in increased production of the pro-inflammatory cytokines TNF α , IL-1 α , and IL-6. These inflammatory cytokines stimulate the production of RANKL in marrow stromal cells, B-lymphocytes, and T-lymphocytes, all of which support osteoclastogenesis. In addition, estrogen deficiency has been shown to result in an increase in IL-7. IL-7 increases T-cell activation, which leads to increased interferon gamma and TNF α that subsequently lead to increased RANKL and osteoclastogenesis (Weitzmann & Pacifici 2005).

Impact on craniofacial bones

Pathologic bone loss around teeth is the hallmark of periodontitis and one of the most common causes of tooth loss. Osteoporosis shares many similarities with periodontitis in that both are highly prevalent,

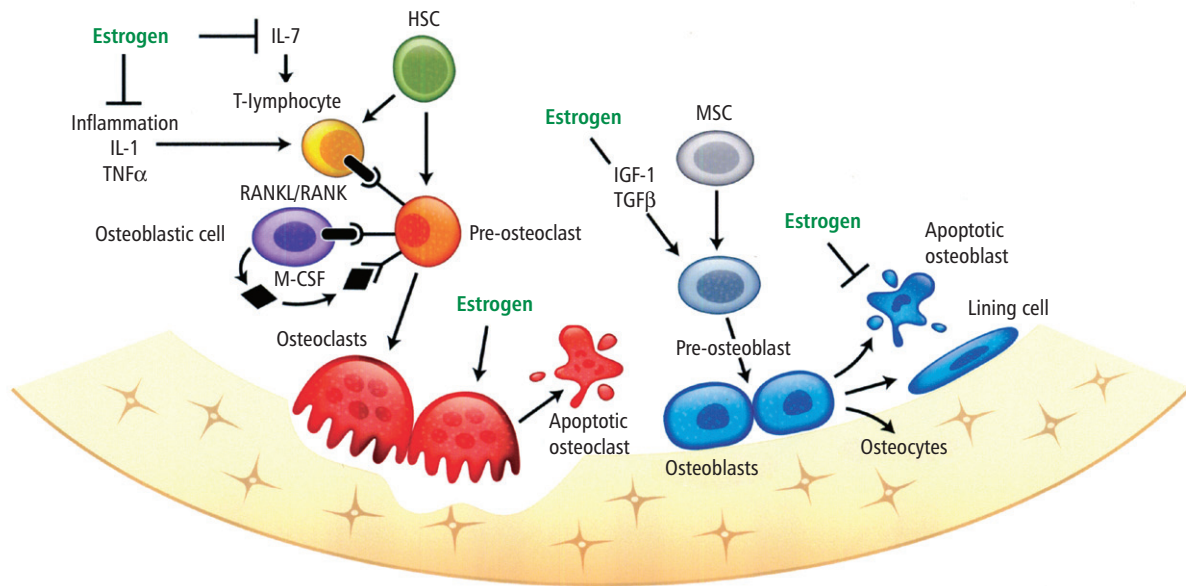


Figure 32.2 Impact of estrogen in the basic multicellular unit. Osteoclasts arise via the differentiation of hematopoietic stem cells along the myeloid lineage and then under the influence of RANKL and M-CSF. RANKL can be presented by osteoblast, stromal, or lymphocytic cells. Osteoblasts arise from mesenchymal stem cells and terminally differentiate into osteocytes or lining cells or undergo apoptosis. Estrogen inhibits the production of inflammatory cytokines (e.g., IL-1, TNF α , and IL-6) that promote RANKL-mediated osteoclastogenesis. Estrogen also blocks the production of IL-7 that mediates T-lymphocytic presentation of RANKL. Relative to bone formation, estrogen promotes the synthesis of IGF-1 and TGF β that stimulates osteoblastogenesis, inhibits the apoptosis of osteoblasts, and hence increases their duration of bone formation.

are marked by frank bone loss, are dependent on host susceptibility, and have multifactorial etiologies. Osteoporosis-associated bone resorption is mediated by similar host immune factors as those seen in periodontal disease, such as inflammatory cytokines. This raises the possibility that estrogen could impact inflammatory cytokines in the oral cavity (Lerner 2006).

There is evidence to support a modest correlation in BMD between bones of the oral cavity and extraoral skeletal sites (Martinez-Maestre *et al.* 2010). However, it is unclear if this decrease in mineralization accelerates the effects of periodontal disease. Due to the multifactorial nature of both conditions, elucidating the connection between these diseases is challenging, and most evidence is based on associations without determining causality. That bones of the craniofacial region are generally intramembranous bones of differing embryologic origin than the traditional osteoporosis target bones (vertebrae, femur, and radius) that form by endochondral bone formation may also support an altered impact of postmenopausal osteoporosis.

Postmenopausal osteoporosis treatments that increase systemic bone mineral density, such as hormone replacement therapy and bisphosphonates, also affect oral bone quality. Increased mandibular BMD is associated with patients taking hormone replacements. While there is

some disagreement in the literature, most studies suggest that oral bisphosphonate use increases bone mineral density, decreases tooth mobility, and improves soft-tissue measures of periodontal disease. In a small percentage of these patients, bisphosphonate-related osteonecrosis of the jaw is a potential side effect warranting caution in these patients despite the overall positive effects in the oral cavity (Kelsey & Lamster 2008).

Dental implant success may be compromised in osteoporosis patients due to decreased bone density, although most studies agree that the bone quality at the implant site is a better predictor of implant outcomes than the patient's overall bone density (Cho *et al.* 2004). Furthermore, there is some evidence that osteoporotic patients experience more bone loss around dental implants over time. While implants are successful in this patient population, these patients may be at a slightly higher risk for implant failure over the long term and would benefit from more frequent recall appointments.

The similarities between periodontitis and osteoporosis have prompted many investigations to determine the relationship between these two diseases. Table 32.1 summarizes the results of these studies, although there is still no consensus on the association between oral and systemic bone loss. Only a few prospective studies have assessed causality between osteoporosis and

Table 32.1 Studies evaluating the relationship between systemic bone mineral density and periodontitis.

	Subject demographics	Dental outcome measure	Systemic outcome measure	Results
Positive relationship between oral and systemic BMD				
(Al Habashneh <i>et al.</i> 2010)	400 PM Jordanian women	Alveolar bone height, CAL, PD, and BOP	Healthy, osteopenic, or osteoporotic as assessed by DXA	Osteoporosis associated with severe alveolar bone loss
(Palomo <i>et al.</i> 2010)	52 PM Caucasian women (28 taking bisphosphonates)	CBCT, plaque score, PD, CAL, BOP, and alveolar bone height	Osteoporosis as assessed by DXA	Bisphosphonate use associated with decreased probing depth independent of plaque score
(Haas <i>et al.</i> 2009)	328 Brazilian premenopausal and PM women classified according to HRT and periodontal status	Periodontitis defined as PAL ≥ 5 mm in $\geq 30\%$ of teeth	Presence or absence of HRT	Greater prevalence of periodontitis in PM women not taking HRT
(Erdogan <i>et al.</i> 2009)	108 PM women	Tooth loss, CAL, PD, and alveolar bone density	Skeletal BMD as assessed by DXA	Low skeletal BMD associated with increased tooth loss, CAL, and alveolar bone density
(Vlasiadis <i>et al.</i> 2008)	141 PM women	Tooth loss, MCW, serum BAP, and NTx	Lumbar spine BMD	Increased MCW and decreased BAP associated with osteopenia and osteoporosis
(Brennan-Calanan <i>et al.</i> 2008)	1256 PM women	Alveolar bone height	Forearm BMD	Increased alveolar bone loss associated with low BMD except in women >70 years
(Gomes-Filho <i>et al.</i> 2007)	139 PM women	Diagnosis of periodontitis or health using clinical examination and radiographs	Densitometry report	Increased incidence of periodontitis in osteoporotic patients
(Yoshihara <i>et al.</i> 2004)	179 Japanese men and PM women	Clinical attachment loss	Healthy or osteopenia	Correlation between PM women and CAL
(Mohammad <i>et al.</i> 2003)	30 PM Asian American women	Tooth loss and CAL	Os calcis BMD	Correlation between BMD and tooth loss and CAL
(Tezal <i>et al.</i> 2000)	70 PM Caucasian women	Alveolar bone height and CAL	Skeletal BMD	Correlation with alveolar bone height and CAL
(Payne <i>et al.</i> 1999)	38 PM women	Alveolar bone height and density	Healthy, osteopenic, or osteoporotic as assessed by DXA	Correlation
(Krall <i>et al.</i> 1996)	189 PM Caucasian women	Tooth loss	Skeletal BMD	Correlation
(Klemetti <i>et al.</i> 1994)	227 PM women	Tooth loss and alveolar bone height	Skeletal BMD as assessed by DXA	Correlation between tooth loss and BMD
(Von Wöwern <i>et al.</i> 1994)	26 PM women	CAL	Forearm BMD	Greater CAL in osteoporotic women
No relationship between oral and systemic BMD				
(Lopes <i>et al.</i> 2008)	47 PM women	GI, PI, and CAL	Healthy, osteopenic, or osteoporotic as assessed by lumbar DXA	No relationship
(Earnshaw <i>et al.</i> 1998)	1365 PM Caucasian women	Tooth loss	Lumbar and proximal BMD	No relationship
(Elders <i>et al.</i> 1992)	286 PM women	Alveolar bone height and tooth loss	Metacarpal cortical thickness and lumbar BMD	No relationship
(Kribbs 1990)	112 PM women	CAL	Health or osteoporosis as assessed by BMD	No relationship

BMD: bone mineral density; PM: postmenopausal; CAL: clinical attachment loss; PD: probing depth; BOP: bleeding on probing; HRT: hormone replacement therapy; MCW: mandibular cortical width; BAP: bone alkaline phosphatase; NTx: cross-linked N-telopeptides of type I collagen; GI: Gingival Index; and PI: Plaque Index.

periodontitis. A seven-year longitudinal study found a significant association between tooth loss and systemic BMD in postmenopausal women (Krall *et al.* 1996). A positive association between T-scores and alveolar bone height and density was reported in another prospective trial of postmenopausal women (Payne *et al.* 1999). Clinical attachment loss (CAL) is a soft-tissue measure of the connective tissue support around teeth. A study of both men and women found a positive correlation between decreased BMD and CAL in postmenopausal women, but not in men (Yoshihara *et al.* 2004).

Correlations between skeletal BMD and number of remaining teeth, alveolar bone height, and alveolar BMD have also been established. Several studies have shown that low systemic BMD is correlated with oral soft-tissue status. In cross-sectional studies, increasing CAL was frequently associated with decreased systemic BMD or osteoporotic status. Positive benefits in the oral cavity have also been reported for osteoporosis treatments such as bisphosphonates and calcium and vitamin D supplementation (Miley *et al.* 2009; Palomo *et al.* 2010).

There is evidence to support that the jaws are not immune to the decrease in bone mineral density that is associated with the skeleton of osteoporotic patients. A direct causal relationship between osteoporosis and periodontal disease has not been well established, and this lack of agreement between studies may be due to differences in the populations studied and the methods used to evaluate the systemic and alveolar bone changes. In addition, there are differences between long bones and those of the oral cavity including the type of loading the bones experience. In the oral cavity, tooth loss further changes the mechanical factors that play a role in accelerating oral bone loss. Decreased bone density in the oral cavity provides a plausible rationale for asserting that osteoporotic patients are at increased risk of alveolar bone loss and may warrant closer management protocols. However, this still is not well established.

Age-associated osteoporosis

Bone quality changes constantly throughout life. The effects of aging on bone composition are reduced bone mass, increased brittleness, and changes in the cortical and trabecular structure. Cortical bone thins and trabecular bone undergoes volume changes. There are gender differences as well. Loss of trabecular bone strength in men is due to thinning of the struts, whereas women experience a loss in connectivity of the struts (Aaron *et al.* 1987).

Osteoblast activity is less effective with increasing age, resulting in defective bone formation. The inability to repair microdamage, combined with an increased

amount of trauma, results in microcrack propagation within the bone. While BMD usually represents an accurate assessment of fracture resistance, it fails to account for the effect of microfractures within bone. Individuals with the same BMD were found to have a 10-fold greater risk of fracture if they were older compared to their younger counterparts (Kanis 2002). Increased presence of microfractures, combined with reduced cortical and trabecular volume and strength, can greatly increase the risk of fracture. As these effects accumulate throughout life, the resulting decrease in BMD is referred to as *age-related osteoporosis*.

Unlike the rapid bone loss associated with postmenopausal osteoporosis, age-related osteoporosis is characterized by a slow and steady increase in bone fragility and brittleness over time. Whereas a sudden decrease in estrogen is the primary etiologic factor in postmenopausal osteoporosis, causes of age-related osteoporosis are numerous, and include hormonal imbalances and impaired bone remodeling. Hyperparathyroidism, decreased sex hormone secretion, nutritional deficiencies including vitamin D, impaired growth hormone secretion, sarcopenia, and leptin and serotonin secretion are all involved in the aging process and implicated in age-related osteoporosis.

Implications in the oral cavity

Bones of the oral cavity experience similar age-related changes to those of the rest of the skeleton. The maxilla and mandible experience a decrease in mineral density and may be more susceptible to periodontal disease when the systemic skeleton is affected by osteoporosis (Boskey & Coleman 2010). While the factors that contribute to oral bone loss are similar to those that affect systemic bone loss, there are several additional etiologic factors present in the mouth that mediate the amount of bone destruction that occurs as a result of age-related osteoporosis. The primary cause of periodontitis is bacterial plaque and, hence, oral hygiene status and oral bacterial composition, which are unrelated to osteoporosis, play a major role in oral bone loss. The host immune response is also critical in controlling oral bacteria; immune dysfunction increases the risk of oral bone loss to a greater extent than systemic bone loss.

Fractures in age-related osteoporosis are due to stress on load-bearing bones that are weakened by decreased BMD. Bones of the oral cavity experience a different type of stress loading through chewing forces, and so these forces affect the weakened bone differently. The mandible experiences more age-related bone loss than the maxilla (Sarajlic *et al.* 2009). Animal models show a relationship between age and oral bone loss that is distinct from that seen in postmenopausal osteoporosis (Liang

et al. 2010). In humans, there is well-supported evidence that mandibular and maxillary bone density correlates with systemic bone density (Table 32.1). Human studies that correlated clinical parameters of periodontitis, such as clinical attachment loss and osteoporosis, were inconclusive.

Since males do not experience the sudden decrease in steroid hormones associated with menopause, this population is ideal for studying the effects of aging on oral bone. There are few studies involving men, and only one that compares the effect of aging between men and women. One study correlated age-related BMD changes with women, but not men (Ronderos *et al.* 2000). In studies with only male subjects, clinical attachment loss has been associated with osteoporosis (Yoshihara *et al.* 2004), whereas other studies failed to find an association (Phipps *et al.* 2007).

Glucocorticoid-induced osteoporosis

Glucocorticoids are potent anti-inflammatory and immunosuppressive drugs commonly prescribed for the management of inflammatory autoimmune conditions, neoplasias, and prevention of posttransplant organ rejection. They are a preferred therapy due to their effectiveness, but they are also associated with a high degree of morbidity. Glucocorticoids increase osteoclastogenesis by reducing osteoprotegerin levels and increasing RANKL. At the same time, glucocorticoids stimulate osteoblast apoptosis and decrease osteoblastogenesis (Canalis 2005). The result is a rapid increase in bone resorption and the establishment of glucocorticoid-induced osteoporosis.

Glucocorticoids are the most common cause of drug-induced osteoporosis. The amount of bone loss is highly variable based upon the dose and duration of glucocorticoids. In general, the greatest change in BMD occurs during the first three months of glucocorticoid therapy, with maximum bone loss occurring at six months (Mazziotti *et al.* 2010). Bone loss continues postglucocorticoid therapy but at a much lower level. This is because the initial phase of glucocorticoid administration has an effect on both osteoclast and osteoblast function. At later stages, the decrease in bone remodeling reaches a steady state and is due to impaired osteoblastogenesis with no significant increase in osteoclast activity.

Risk factors for glucocorticoid-induced osteoporosis include smoking, low body mass index, and high daily or cumulative glucocorticoid doses. In addition, individuals respond differently to the same steroid dose; however, the degree of bone loss is positively correlated to the dose and duration of the medication. Additional medications are often prescribed to treat the unwanted

secondary effects of glucocorticoid administration. Historically, calcitonin was used to counteract glucocorticoid-induced bone loss. Today, oral bisphosphonates are the most common treatment.

Implications in the oral cavity

Severe oral bone loss associated with glucocorticoid-induced osteoporosis can be very debilitating. Few human studies have looked at the oral effects of glucocorticoid therapy, but animal studies reveal that glucocorticoids have a profound catabolic effect in the oral cavity. Histopathologically, the mandible and maxilla in patients treated with glucocorticoids are characterized by bone loss in the interdental septum, reduced cellularity in the periodontal ligament space, wide osteocyte lacunae, disorganized Haversian canals, irregular bone trabeculae, and large medullary cavities. Like most types of osteoporosis, the trabecular bone is more affected than the cortical bone. A substantial decrease in the amount of alveolar bone trabeculae in glucocorticoid-induced osteoporotic patients has been reported.

Bisphosphonates and calcitonin are sometimes prescribed to offset the catabolic effects of the glucocorticoids. An animal study compared the ability of two osteoporotic medications, calcitonin and alendronate, to prevent oral bone loss in rats with glucocorticoid-induced osteoporosis (Ezzat 2011). Both therapies were effective at reducing the oral effects of glucocorticoid use with alendronate outperforming calcitonin. However, these treatments raise a further concern in this patient population because of the added risk of osteonecrosis of the jaw that is associated with the use of bisphosphonates.

In conclusion, glucocorticoid-induced osteoporosis is associated with rapid bone loss including the oral cavity. Little is known about the association between oral and systemic bone loss in these patients, or treatment options to minimize these effects. Several animal studies highlight the potential for the use of anti-osteoporotic drugs in the prevention of drug-induced oral bone loss, but human studies are lacking.

Osteoporosis secondary to cancer: androgen ablation and aromatase inhibitors

Primary osteoporosis is bone loss associated with natural aging and the decline of gonadal function in both men and women. Secondary osteoporosis is due to accelerated bone loss precipitated by an identifiable drug (glucocorticoids or anticonvulsant therapy), disease process (hypoestrogenemia), or nutritional deficiency. Breast and prostate cancers account worldwide for 23% of cancer cases in women and 12% of cancer cases in men.

Surgical gonadal ablation and endocrine cancer therapies such as androgen deprivation therapy and aromatase inhibitors that target hormone levels place both men and women at risk for osteoporosis.

Androgen deprivation therapy (ADT) has been shown to have a clear association with osteoporosis in men, although both the strength and the significance of this association appear to be functions of the duration of ADT (Taylor *et al.* 2009). Men treated for prostate cancer with either castration or gonadotropin-releasing hormone agonists demonstrate a significantly increased risk for lower BMD (Taylor *et al.* 2009). Studies have shown rates of bone loss ranging from 2% to 8% in the lumbar spine and from 1.8% to 6.5% in the femoral neck during the initial 12 months of continuous ADT (Oefelein *et al.* 2001). ADT also increases the risk for fracture with a greater than six-year duration of therapy (Taylor *et al.* 2009).

For women diagnosed with breast cancer, nearly 70% of these cancers express estrogen receptors and therefore are amenable to adjuvant endocrine therapy. Aromatase inhibitors (AIs) inhibit estrogen synthesis, thereby decreasing circulating estrogen levels by more than 90% (Forbes *et al.* 2008). As estrogen levels are lowered substantially below postmenopausal levels, increased bone turnover and bone loss raise the relative risk for osteoporosis, osteopenia, and fractures (Forbes *et al.* 2008). In the Anastrozole, Tamoxifen, and Combination Trial, an average of 7%–8% loss of bone mineral density (BMD) per year and an increased risk of fracture were noted over the course of five years in the anastrozole-treated group (Oefelein *et al.* 2001). Comparative studies of AIs suggest that their effect on fractures and bone loss are similar across the class of drug (Taylor *et al.* 2009). Not surprisingly, women with baseline osteopenia are more likely to develop osteoporosis while on AI therapy than are women with normal baseline BMD. Recent data suggest that accelerated bone loss associated with AIs ends once therapy is discontinued (Taylor *et al.* 2009).

Implications in the oral cavity

Patients undergoing endocrine-related cancer treatment are at increased risk for oral complications due to reduced bone mass, and development of osteoporosis as a result of their cancer treatments. Periodontal diseases may be influenced by systemic bone loss (Dervis 2005). Imbalanced bone remodeling associated with the use of AIs and ADT has demonstrated a net loss of bone density throughout the skeleton. Skeletal mass reduction may include the craniofacial bones, particularly the mandible (Santen 2011). Studies have shown that bone changes in osteoporosis are associated with the loss of periodontal attachment, loss of teeth, and reduction of the height of

the residual ridge (Dervis 2005). Furthermore, osteoporosis of the mandible and maxilla are associated with a greater risk for loss of teeth and/or implant failure. Based on these findings, it has been hypothesized that osteoporosis could be a risk factor for the progression of chronic periodontitis in patients receiving endocrine-related cancer treatment.

Using a cross-sectional study design, Famili and colleagues (2007) examined periodontal disease and ADT in men undergoing ADT therapy (Famili *et al.* 2007). Periodontal disease was three times more prevalent in patients receiving ADT, but after adjustment for age, race, smoking, and periodontal treatment history, the relationship did not reach statistical significance. There was no correlation between BMD, periodontal disease or tooth loss, and ADT (Famili *et al.* 2007). Prospective studies composed of patients beginning ADT are necessary to delineate the relationship between ADT and oral health.

Presently it is not known if AIs have an impact on oral health. Mechanistically, since estradiol (estrogen) depletion has been shown to be an important risk factor for the development of osteoporosis and AIs deplete estrogen to nearly nonmeasurable levels, a theoretical risk exists and therefore represents a possible risk factor for oral conditions among postmenopausal women.

Despite the recognition of the consequences of ADT and AI therapy related to low BMD, osteoporosis, and fractures, there is limited information at the present time on the implications of endocrine cancer therapy on oral health.

Primary hyperparathyroidism

Primary hyperparathyroidism is a relatively common endocrine disorder with an incidence of nearly 1 in 1000 adults. It occurs most frequently in the third to fifth decade of life and affects females more frequently than males with a 3:1 ratio (Fraser 2009). The parathyroid glands, located in the neck region, are responsible for producing parathyroid hormone (PTH) in response to fluctuations in serum calcium that trigger the calcium-sensing receptor in the parathyroid gland. PTH functions to regulate serum calcium by stimulating calcium release from the bone (i.e., activating osteoclastic resorption) and conserving calcium excretion by the kidney (Figure 32.3). In bone, PTH binds to receptors on osteoblasts that signal primarily through the PKA and PKC pathways. PTH promotes osteoblast expression of RANKL that stimulates bone resorption to provide calcium release. PTH is also anabolic when administered therapeutically for the stimulation of bone formation via indirect and as yet unclear effects on osteoblasts.

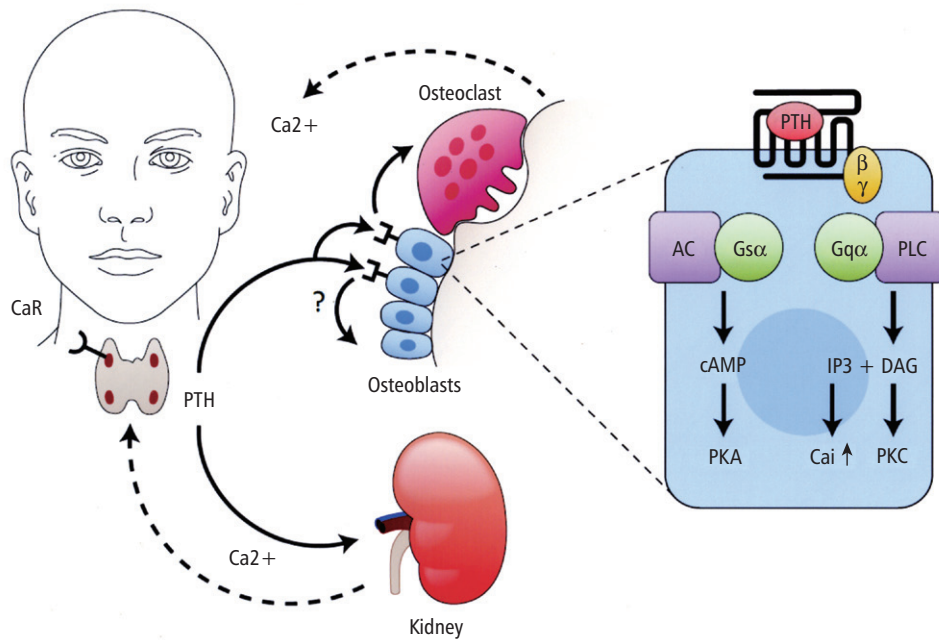


Figure 32.3 Parathyroid hormone (PTH) is an 84–amino acid protein synthesized by the parathyroid glands. PTH circulates to the target organs of bone and kidney where it acts to maintain serum calcium levels by increasing calcium release from the bone and inhibiting calcium excretion from the kidney. PTH binds to G-protein-linked 7 transmembrane domain receptors in osteoblasts and signals through the second messengers cAMP/PKA and PKC. PTH secretion is modulated by circulating calcium as it interacts with the calcium-sensing receptor (CaR) in the parathyroid gland in a feedback loop.

The etiology of primary hyperparathyroidism is a solitary adenoma of the parathyroid gland in 80% of cases. Other less common causes include hyperplasia of the gland or carcinoma. Primary hyperparathyroidism often presents with no symptoms and is found incidentally on routine blood calcium screening as asymptomatic hypercalcemia. The most common clinical signs include renal stones, subperiosteal erosions, diffuse osteoporosis, brown tumors, “salt-and-pepper” mottling of the skull, and gastrointestinal discomfort. Surgical removal of the adenoma results in rapid amelioration of hypercalcemia and symptoms. Normocalcemic hyperparathyroidism has also emerged as a variant with little symptomatology (Lowe *et al.* 2007).

Implications in the oral cavity

The classical literature describes clinical signs of primary hyperparathyroidism in the oral cavity as brown tumors and widened lamina dura. Brown tumors are not actual tumors but represent focal areas of increased bone resorption that are filled with fibrous tissue, woven bone, and vasculature. The term *brown* comes from the deposition of hemosiderin in the site. These lesions appear as radiolucent areas radiographically; however, their appearance is actually quite uncommon as diagnostic identification of primary hyperparathyroidism is occurring much earlier in the disease progression than it was

50 years ago. Histologically, brown tumor lesions appear largely as granulation tissue with numerous osteoclast-like giant cells. A genetic variant of primary hyperparathyroidism is hyperparathyroid jaw tumor syndrome (HPT-JT), which has been attributed to mutations in the HRPT2 gene (Aldred *et al.* 2006). These patients present with radiolucent lesions in the jaw that histologically resemble cemento-ossifying fibromas and typically lack the presence of giant cells. The classical description of widened lamina dura in hyperparathyroidism, which represents a manifestation of subperiosteal bone loss, is rare. Manifestations such as these are more likely in patients with severe or prolonged disease. Instead, signs of an anabolic-like activity of PTH have been reported that include increased incidence of tori in the oral cavity of patients with primary hyperparathyroidism (Padbury *et al.* 2006).

Secondary hyperparathyroidism and renal osteodystrophy

Secondary hyperparathyroidism occurs when the parathyroid gland is stimulated to overproduce PTH in response to low serum calcium levels. Low serum calcium levels occur in a variety of conditions that impact calcium intake and phosphate metabolism. Most common examples include low dietary calcium, excessive urinary

calcium excretion, vitamin D disorders, malabsorption, malnutrition, and kidney disease.

The term *renal osteodystrophy* describes various skeletal pathologies; *chronic kidney disease mineral bone disorder* (CKD-MBD) is a more recent term that encompasses renal osteodystrophy as well as the broader syndrome of mineral metabolism and cardiovascular and skeletal disorders (Svara 2009). Hyperphosphatemia is central to most aspects of these conditions with compromised renal function resulting in reduced filtration of phosphate. Increased circulating phosphate results in reduced serum calcium through physiochemical binding. Hyperphosphatemia also results in suppressed 1 alpha hydroxylase (an enzyme essential for vitamin D activation), which results in lower calcitriol levels. Hyperphosphatemia is associated with heterotypic mineralization of the vasculature and hence the multi-organ nature of these disorders. Hyperphosphatemia can also lead to direct stimulation of the parathyroid gland resulting in diffuse cellular hyperplasia and increased PTH production. A sustained increase in PTH will lead to abnormal osteoblast function with less type I collagen production, increased RANKL, and increased FGF23. FGF23 inhibits mineralization. The histologic picture is that of increased osteoblasts, osteoclasts, and osteocytes but a disorderly collagen and increased osteoid.

Implications in the oral cavity

In the oral and craniofacial region, renal osteodystrophy presents as radiographic signs of osteitis fibrosa, osteomalacia, loss of radicular lamina dura, and a diffuse “ground glass” appearance. Jaw enlargement is a rare complication that may occur in a localized or more generalized pattern. Patients with chronic kidney disease have been reported to have increased severity of periodontal disease as well as increased red-complex bacteria and *Candida albicans* (Bastos *et al.* 2011). Dental and periodontal health has been found to worsen with time for patients on hemodialysis for the treatment of chronic kidney disease (Cengiz *et al.* 2009). The likelihood of a bidirectional effect of the chronic inflammation associated with periodontal disease impacting kidney function as well as the uremic nature of chronic kidney disease impacting gingival inflammation is strong (Fisher *et al.* 2011).

Paget’s disease

Paget’s disease of bone (PDB) is a metabolic bone disease characterized by increased bone resorption coupled with excessive and unregulated bone formation, which can affect a single bone or multiple bones. This results in weakened and deformed bones of increased mass in

which the collagen fibers assume a haphazard and irregular mosaic pattern devoid of symmetry. The bones most commonly affected by this condition include the pelvis (70%), femur (55%), lumbar spine (53%), skull (42%), and tibia (32%) (Noor & Shoback 2000).

Aside from osteoporosis, Paget’s disease is the most common bone disorder (Noor & Shoback 2000). PDB may or may not be symptomatic and is frequently detected when radiographs are obtained for other reasons or because routine blood screening detects a high level of alkaline phosphatase. The most common symptom is pain in the affected bone, although neurologic, hearing, vision, cardiac, and oncologic complications are possible. Paget’s disease is equally prevalent in men and women, with increased incidence in persons older than 40 years of age. It affects approximately 3%–4% of the population in the United States and as many as 10% of people older than 80 years (Noor & Shoback 2000).

Prevalence varies considerably by geographic location; the condition is rare in the Far East and most common in the northwest of England. A marked reduction in both the incidence of new cases and the extent of bone involvement of Paget’s disease has been reported. While the disease is becoming less common, it is uncertain what changes in the environment or what exposure to causal agents or changes in ethnicity is driving the reduced incidence of Paget’s disease (Langston & Ralston 2004).

Paget’s disease etiology

Paget’s disease presents as an abnormality in osteoclasts with increased numbers of cells and increased size and numbers of nuclei per cell. Increased bone resorption occurs with an attempt for coupling by increased osteoblast activity. The result is increased bone turnover with associated increases in biochemical parameters, increased vascularity near Pagetic lesions, and mixed radiolucent and sclerotic lesions.

The etiology of Paget’s disease remains largely enigmatic with several proposed pathogenic mechanisms (Roodman 2010). The geographic distribution and familial pattern of Paget’s disease suggest a genetic predisposition. Three altered gene profiles have been proposed as being associated with Paget’s disease. Mutations in RANK have been proposed but have not been found to be widespread in Paget’s patients. A mutation in the gene encoding sequestasome-1, a ubiquitin binding protein that plays a role in NFκB signaling, has been found in approximately 30% of Paget’s patients, but mutations have also been reported in normal patients. Mutation of this gene in mice has failed to reproduce the Paget’s phenotype. A viral etiology is the other widely

discussed pathogenesis of Paget's disease. Both the measles virus and the canine distemper virus have been investigated for their presence and impact on osteoclast function. The role of such viral inclusions in Pagetic osteoclasts and their ability to associate with the osteoclastic Pagetic phenotype are still controversial. The likelihood of a combined genetic and environmental etiology that incorporates both the familial nature and the potential for inciting factors in the development of PDB is strong.

Implications in the oral cavity

Approximately one fifth of patients with Paget's disease have oral manifestations, with the maxilla most often affected (Kelsey & Lamster 2008). Paget's disease is characterized by high bone turnover, which causes oral clinical features such as facial deformities, malocclusion, spreading of teeth, and poorly fitting dentures. The radiographic appearance of the jaws results from excessive bone deposition; it is typically described as having a cotton wool or ground glass appearance.

Dental complications arise from abnormal circulation and altered bone formation (Woo & Schwartz 1995). In early stages of the disease, multiple arteriovenous (AV) shunts form. This greatly increases the chance of excessive bleeding during routine oral surgery procedures such as dental extractions and alveoplasty. Poorly vascularized, sclerotic regions of bone form during later stages of the disease; this predisposes the patient to chronic infection and osteomyelitis. In extreme cases, the increased bone turnover is associated with nerve compression and neuropathy. In addition, the newly formed bone is of poor quality, and spontaneous pathologic fractures and osteonecrosis have been reported.

The most common treatment for Paget's disease is oral or intravenous bisphosphonates. The doses used to treat Paget's disease are higher than for osteoporotic patients, but less than for cancer patients. As a result, patients with Paget's disease may be at increased risk of developing osteonecrosis of the jaw (ONJ) due to decreased vascularity and poor bone quality. The estimated prevalence of ONJ in patients with Paget's disease is 0.01%–0.04% (Vescovi & Nammour 2011).

Summary

Bones of the craniofacial region share features of metabolic bone disease with other skeletal sites, but they also have unique features and, in many cases, as yet unclear features relative to systemic bone disease. That the osteoblast, osteoclast, and osteocyte cells comprising the craniofacial skeleton are phenotypically similar at various skeletal sites suggests craniofacial bones should respond

similarly to systemic changes. However, that these bones are of differing embryologic and developmental origin and experience different environmental states (biomechanics and microbial impacts) supports their unique response to metabolic influences. A fundamental understanding of the cellular-, tissue-, and organ-level changes that endocrine mediators use to impact various skeletal sites renders the clinician better able to provide optimal patient care, and the scientist better able to ask pertinent and significant questions to expand our knowledge and inform appropriate clinical care.

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Clinical correlate: renal osteodystrophy

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Renal osteodystrophy is the bone pathology associated with chronic kidney disease (CKD).

Renal osteodystrophy is part of a clinical syndrome called chronic kidney disease–mineral and bone disorder (CKD-MBD). This syndrome, as the name suggests, describes the systemic mineral and bone metabolism disorders due to chronic kidney disease. It is manifested by either one or a combination of the following: a) abnormalities of calcium, phosphorous, PTH, or vitamin D metabolism; b) abnormalities in bone turnover, mineralization, volume, linear growth and strength, and; c) vascular or other soft tissue calcification. (Moe *et al.* 2006)

In renal osteodystrophy, the dysregulation in bone remodeling can result in increased or decreased bone turnover. Radiographically, loss of lamina dura, “ground glass” appearance of the bone, and loss of trabeculation are often observed. The classic histological features of renal osteodystrophy are osteitis fibrosa, increased bone remodeling, and fibrosis of the marrow. In addition, patients may have enlargement of the jaws (Damm *et al.* 1997; Joy *et al.* 2007). This chapter describes a patient with jaw enlargement due to renal osteodystrophy. The surgical management of this patient was directed toward improving his masticatory function and appearance by osseous recontouring and decreasing the bony expansion of the affected maxillofacial region. A parathyroidectomy was performed for the management of his secondary hyperparathyroidism.

Case presentation

In 2006, a 45-year-old male was referred to the Oral Surgery Department at the University of California, Los

Angeles (UCLA), School of Dentistry for treatment of maxillary hyperplasia. The patient’s chief complaints were facial swelling and difficulty eating. The patient also stated that the swelling started one year prior to his visit. The patient’s medical history revealed a familial history of kidney disease. He presented with end-stage renal disease, and was treated with a renal transplant in 1995 that failed in 2005. The patient was on hemodialysis three times a week. In addition to the end-stage renal failure, the patient had secondary hyperparathyroidism, diabetes mellitus, hypertension, hyperlipidemia, coronary artery disease, and depression. His medications included almodipine, atorvastatin, lisinopril, carvedilol, insulin injections, metoclopramide, and temazepam. The patient denied tobacco, alcohol, and recreational drug use.

Clinical assessment revealed expansion of his midface and mandible with splaying of teeth, decreased airway space, and bleeding. The clinical differential diagnosis was renal osteodystrophy, Paget’s disease, and fibrous dysplasia. The radiographic assessment revealed loss of lamina dura and a decrease in bone trabeculation characterized by a “ground glass” appearance. A biopsy taken from the maxillary anterior alveolar ridge revealed a fibrous lesion. Bone trabeculae were present amongst fibrous tissue, multinucleated-osteoclast-type giant cells were present within the lesion, and osteoblastic rimming was almost absent.

The final diagnosis was based on his history and clinical presentation, which was consistent with osteitis fibrosa cystica due to renal osteodystrophy (CKD-MBD). At that time, the treatment option was to correct the secondary hyperparathyroidism, since in many cases this leads to a decrease in the facial overgrowth. If necessary,

the patient would also undergo surgery to correct or decrease facial abnormalities.

In September 2008, at the age of 47, the patient returned to the Oral Surgery Department at the UCLA School of Dentistry. By then, enlargement of the patient's jaw had increased, and he had even more difficulty eating and speaking. Moreover, the patient reported weight loss, fatigue, and malaise. He was also wheelchair bound, secondary to other manifestation of his disease, which included lower- and upper-extremity swelling.

The patient's medical history and medications remained similar to the ones reported in 2006. He was given an additional diagnosis of CKD-MBD. Due to his inability to respond to treatment, the patient was scheduled for a total parathyroidectomy and re-implantation of one-half parathyroid. Prior to surgery, serum levels of parathyroid hormone (PTH) levels were as high as 5837 pg/ml (the average is 10–60 pg/ml). See detailed laboratory results in Table 33.1.

The clinical assessment revealed mandibular and maxillary overgrowth (Figure 33.1). The nasal cavity was obliterated by tissue overgrowth. The patient had intra-oral gingival swelling associated with bony overgrowth in both the maxilla and mandible with mobility and splaying of the teeth (Figure 33.1). The patient was a Mallampati Class 4 with posterior airway space narrowing due to hypertrophy of the soft and hard tissues. The radiographs revealed a loss of lamina dura and a decrease in bone trabeculation characterized by a "ground glass" appearance (Figure 33.2). The surgical treatment plan was osseous reduction and recontouring of the maxilla including the extraction of teeth due to severe mobility. Surgery was recommended because of the patient's inability to eat and to facilitate his breathing.

The patient underwent osseous reduction and recontouring in conjunction with total parathyroidectomy and re-implantation of one-half a parathyroid into the sternocleidomastoid (Figures 33.3 and 33.4). At first, two parathyroids were removed and PTH levels were analyzed (Table 33.1). Subsequently, the remaining two parathyroids were removed and one-half a parathyroid was re-implanted into the sternocleidomastoid (Table 33.1). The patient suffered no major complications from the maxillary reduction. However, due to the parathyroidectomy, the patient developed hypocalcemia and hypophosphatemia in addition to vitamin D deficiency. After the surgery, the patient's medications were changed due to the hypocalcemia and hypophosphatemia. His medications included calcium carbonate, almodipine, ascorbic acid, aspirin, lipitor, calcitriol, calcium carbonate, calcium gluconate, varvedilol, ergocalciferol, iron sulfate, gabapentin, hydralazine, dilaudid, insulin, magnesium sulfate, and zinc sulfate. Laboratory results at the time of discharge are seen in Table 33.1.

Discussion

This chapter describes a patient with CKD-MBD or, more specifically, renal osteodystrophy that included jaw enlargement. Renal osteodystrophy represents skeletal complications to chronic kidney disease with a wide histological presentation. The diagnosis and treatment of renal osteodystrophy are often monitored by using PTH as a surrogate indicator of bone turnover in conjunction with serum levels of calcium phosphate and calcitriol. Nonetheless, bone biopsy, with subsequent histological analysis and clinical correlation, is the preferred method of diagnosis. Histologically, renal osteodystrophy can

Table 33.1 Laboratory tests performed prior to, during, and after surgery.

	11/2007	Right before the surgery (9/2008)	During the surgery after removal of two parathyroids	During the surgery after removal of 3.5 parathyroids	At discharge	Reference range
Phosphorous	7.3	4.1			2.3	2.3–4.5 mg/dl
Total calcium	8.9	9.1				8.4–10.2
Ionized Calcium (corrected)		1.26			1.16	1.09–1.29 nmol/L
PTH	5837	3354	939	204	33	11–60 pg/ml
Alkaline phosphatase	1245					40–129 U/L
Glomerular filtration rate (estimate)		12			16	Kidney failure (<15)



Figure 33.1 Clinical photos at presentation. A. Frontal view. B. Lateral view of the bilateral expansile mandibular and maxillary lesions. C. Intraoral view of the palatal enlargement.

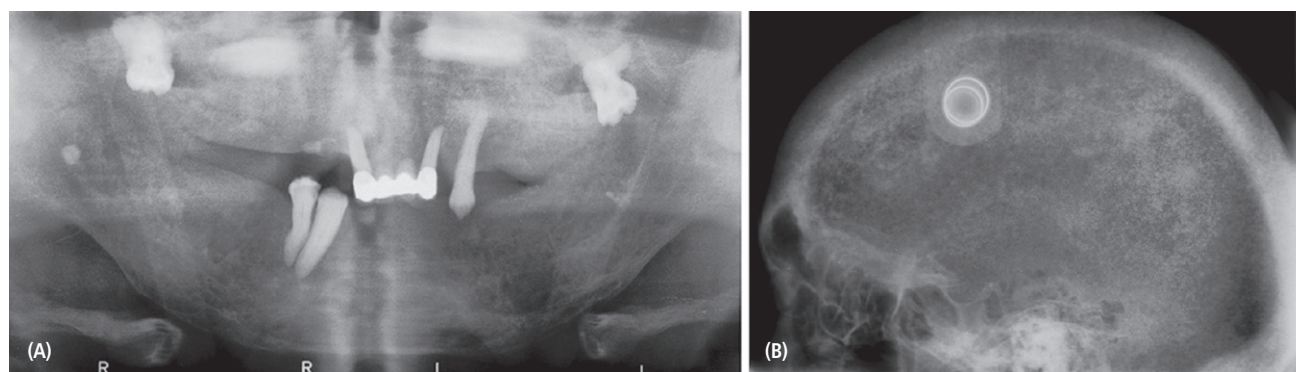


Figure 33.2 Radiographs at presentation. A. Panoramic radiograph. B. Lateral skull radiograph demonstrating the "ground glass" appearance of the trabeculae.

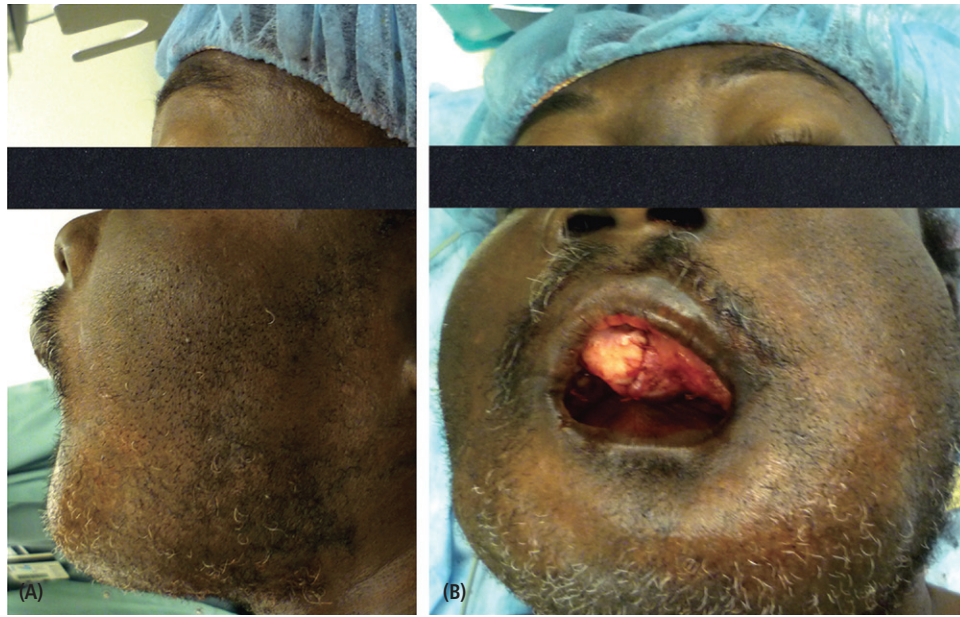


Figure 33.3 Clinical photos of patient taken immediately after surgery. A. Lateral view. B. Frontal view.

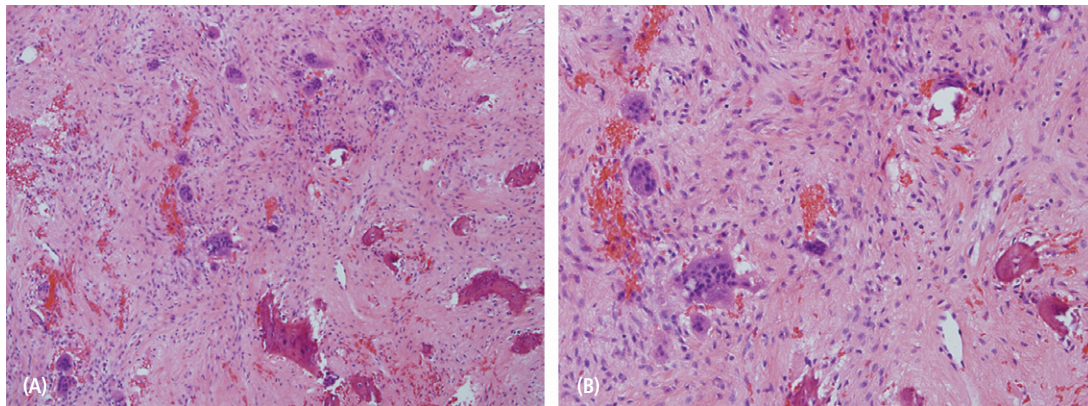


Figure 33.4 Histological photos. A. Hematoxylin and eosin (H&E) staining of a biopsy section with low-power magnification. B. H&E staining of a core biopsy section with high-power magnification. Several large multinucleated osteoclastic cells are present.

manifest as osteitis fibrosa, osteomalacia, or a combination of both. The classic histological feature of renal osteodystrophy is osteitis fibrosa, which arises from increased remodeling and fibrosis of the marrow. Osteomalacia can also be observed when there are decreased PTH levels as a consequence of parathyroidectomy or overtreatment with vitamin D. In osteomalacia, there is a decrease in bone turnover resulting in reduced bone volume, mineralization, and collagen synthesis. Additionally, there is a decrease in the ability of the skeleton to buffer serum calcium changes. It is important to note that the histological presentation of renal osteodystrophy can go from one form to the other (Coen *et al.* 2002;

Freemont & Malluche 2005; Malluche & Monier-Faugere 2006; Joy *et al.* 2007).

Jaw enlargement due to renal osteodystrophy is a rare condition and may resemble fibrous dysplasia and Paget's disease. Fibrous dysplasia is characterized by the replacement of bone by a fibrous connective tissue and affects the maxilla more than the mandible. Radiographically, it has a "ground-glass" appearance. However, fibrous dysplasia is usually diagnosed by the second decade of life and the biochemical profile is normal, which is not the case with renal osteodystrophy (Neville *et al.* 2002). In the case of Paget's disease, which mostly affects older adults, serum alkaline phosphatase is ele-

vated but calcium and phosphorous levels are normal. Therefore, the alkaline phosphatase levels in Paget's disease and renal osteodystrophy are similar (Damm *et al.* 1997; Adornato & Mayne 2000). According to Damm and colleagues (1997), any fibrous lesion in the jawbone in a patient with end-stage renal disease must be considered renal osteodystrophy until proven otherwise (Damm *et al.* 1997; Adornato & Mayne 2000; Malluche & Monier-Faugere 2006). The patient presented with end-stage renal disease and post-renal transplant failure. The PTH levels reached 5837 pg/ml prior to maxillofacial recontouring and parathyroidectomy. Consequently, the history of chronic renal disease and renal failure confirmed the diagnosis as renal osteodystrophy.

The treatment for renal osteodystrophy is correction of the secondary hyperparathyroidism. The correction of secondary hyperparathyroidism initially includes restricted diet (phosphate) and the use of phosphate-binding agents and active vitamin D (calcitriol). If hyperparathyroidism is not resolved with diet and medications, it may require a parathyroidectomy and/or renal transplantation (Malluche & Monier-Faugere 2006). The patient discussed in this chapter was on a restricted diet and medications, was receiving hemodialysis, had undergone a renal transplant that unfortunately failed, and was later subjected to total parathyroidectomy and re-implantation of one-half of a parathyroid.

The treatment of the jaw enlargement due to CKD-MBD can sometimes be accomplished by restoring PTH levels (Phelps *et al.* 1994). This usually can be accomplished by having the patient undergo a parathyroidectomy and renal transplant (Neville *et al.* 2002). However, in some cases further surgical treatment is necessary, especially when the facial deformities are so severe that they lead to dysphagia and respiratory difficulties (Dantas *et al.* 1991; Damm *et al.* 1997). This patient had severe extra- and intraoral enlargement and a renal transplant failure. To manage his secondary hyperparathyroidism, a parathyroidectomy was performed. In conclusion, it is important to correctly diagnose jaw enlargement due to renal osteodystrophy and to ensure that the patient receives adequate treatment to decrease potential further complications such as respiratory and nutritional problems.

Summary

Renal osteodystrophy is the bone pathology associated with chronic kidney disease. In the oral cavity, a loss of lamina dura, "ground glass" appearance of the bone, a loss of trabeculation, and brown tumor formation are often observed. Additionally, there is occasionally an enlargement of the jaws. This chapter describes one patient with renal osteodystrophy resulting from end-stage renal failure. The treatment of this patient involved surgery to decrease the maxillofacial bony enlargement and parathyroidectomy to manage the secondary hyperparathyroidism.

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Mineral metabolism and its impact on craniofacial bones and teeth

Jian Q. Feng and Chunlin Qin

Minerals (including calcium, phosphorus, magnesium, potassium, sodium, and iron) are essential for body functions of cells, tissues, and organs. Minerals are also critical for building both soft and hard tissues. Metabolism is the physical and chemical processes within the body that maintain life via creating and using energy. *Mineral metabolism disorders* refer to abnormal levels of minerals—either too high or too low—in the blood caused by genetic defects or by nutritional problems related to starvation, diarrhea, or kidney diseases associated with failure to excrete minerals. In this chapter, we will focus on only calcium and phosphate due to the following two factors: (1) the impact of a disturbance in calcium and/or phosphate homeostasis is much greater on the health of bones and teeth than the impact of any other mineral, and (2) the impact of disorders linked to other minerals that are related to the health of bones and teeth is largely unclear and less studied.

Our understanding of calcium and phosphorus homeostasis began with the discovery of rickets, which is a disorder of children caused mainly by a vitamin D deficiency (Bouillon *et al.* 2008). The first description of this disease dates back 400 years, although the identification and synthesis of vitamin D, by Adolf Windaus, did not occur until the late 1920s. For this discovery, Windaus won the Nobel Prize in Chemistry in 1928 (Wolf 2004). Because calcium is limited in certain foods and phosphorus is present in almost all foods, much of the past research efforts have focused on calcium metabolism. Understanding the controllers of phosphate homeostasis has lagged behind that of calcium homeostasis.

This chapter will briefly summarize the regulation of calcium homeostasis and the impact of calcium homeo-

stasis disorders on bones and teeth. However, the chapter will focus on the recent progress in our understanding of phosphate homeostasis.

Calcium homeostasis and its impact on craniofacial bones and teeth

Calcium distribution

Calcium exists in the form of either its free ion or bound complexes. The key function of calcium in bones and teeth is mineralization. The vast majority of total body calcium (>99%) is present in the bone and tooth as bound complexes (primarily as calcium–phosphate complexes), which are responsible for much of the material properties of hard tissues (i.e., providing strength). Bone, unlike other hard tissues, also provides a dynamic calcium store for daily calcium needs in the body with an exchange rate between bone and the extracellular fluid of approximately 500 mmol of calcium per day. There are three forms of calcium: (1) free ions (50%), (2) protein-bound complexes (albumin and globulin: 40%), and (3) ionic complexes (calcium phosphate, calcium carbonate, and calcium oxalate: 9%). Because the ionized calcium determines the biologic effect of calcium on the extra- and intracellular signaling, nerve impulse transmission, and muscle contraction, the concentration of serum-ionized calcium is precisely maintained within a physiologic range of 4.4–5.4 mg/dl (1.10–1.35 mM).

Calcium balance

Calcium balance, equilibrium over long periods of time (days, weeks, or months), is maintained by the net effect

of intestinal absorption; the excretion of calcium from renal, intestinal, and sweat glands; and calcium fluxes in and out of bone (bone balance). Bone balance (formation and resorption) changes throughout the life span, which is a key factor in the determination of calcium balance. In children, both bone formation and resorption remain at high levels, although bone formation is greater than bone resorption (positive bone balance) to ensure normal skeletal growth. In young adults, bone formation is equal to bone resorption (net bone balance), and achieves peak bone mass. In elderly adults, bone formation is less than bone resorption (negative bone balance), which leads to age-related bone loss (osteoporosis). Factors favoring positive bone balance are exercise and anabolic and antiresorptive drugs; factors negatively affecting bone balance include immobilization, weightlessness, and sex steroid deficiency.

Regulation of calcium homeostasis

Calcium homeostasis is tightly controlled by an integrated hormonal system that controls calcium fluxes between the kidney, bone, and gut via extracellular fluid or serum. It is regulated by (1) parathyroid hormone (PTH, an 84-amino acid peptide that is synthesized by the chief cells of the parathyroid gland); (2) the PTH receptor (PTHr) expressed in both proximal tubular epithelial cells and bone cells (Amizuka *et al.* 1997; Schipani & Provot 2003); (3) $1,25(\text{OH})_2\text{D}_3$, the vitamin D receptor (VDR; Bouillon 2009); and (4) serum ionized calcium, the calcium-sensing receptor (CaR; Riccardi & Brown 2010).

A decrease in serum calcium first inactivates the CaR in the parathyroid glands to increase parathyroid cellular proliferation, PTH synthesis, and secretion. PTH then targets the PTHr in renal tubules to increase tubular calcium reabsorption. In bone, PTH activates osteoclasts via osteoblasts to increase net bone resorption. However, when given intermittently, PTH is anabolic. In addition, PTH also increases $1,25(\text{OH})_2\text{D}_3$ production in the kidney, which then will act on the VDR in the intestine to accelerate calcium absorption. Conversely, an increase in the ambient calcium will inhibit PTH secretion and accelerate calcium excretion. Furthermore, an increase in calcium will stimulate secretion of calcitonin, a 32-amino acid peptide that is synthesized in the parafollicular cells of the thyroid gland. Calcitonin then will directly inhibit osteoclastic bone resorption to reduce the release of calcium from the bone, although this effect is rapid and may have little effect on chronic calcium homeostasis. Any failure in the regulation of calcium homeostasis will lead to abnormally low or high serum concentration.

Disorders of calcium homeostasis and their effects on craniofacial bones and teeth

As described in this chapter, blood calcium levels are maintained within a tight range due to the fact that almost all physiologic processes depend, in one way or another, on calcium. Deviations either below (hypocalcemia) or above (hypercalcemia) the normal range will result in serious diseases. The pathological causes could be parathyroid problems, vitamin D deficiency, or kidney dysfunction.

Rickets and the development of craniofacial bones and teeth

The most striking craniofacial rickets phenotype is frontal bossing (enlarged forehead) and a box-like shape of the head. Vitamin D, 1α -hydroxylase deficiency or VDR resistance during tooth development affects amelogenesis, dentinogenesis, and cementogenesis. Enamel maturation and mineralization are sharply decreased, leading to a lifelong irreversible enamel dysplasia (Bouillon *et al.* 2008). Furthermore, in children who have rickets, the dentin is thin, and the pulp chamber is enlarged. These rachitic teeth are also more prone to periodontal abscesses. A small-scale clinical trial of vitamin D and calcium supplementation has provided promising results (i.e., reduction of tooth loss in osteoporotic individuals; Hunt & Johnson 2007), although further studies are required to support these data. Note that rickets caused by nutritional factors is rarely seen due to our understanding of its causes and the resultant improvements in diets, including calcium- and vitamin D-fortified foods.

Hypocalcemia indicates a low blood calcium concentration and is associated with increased neuromuscular excitability, including muscle spasms, tetany, and cardiac dysfunction, although chronic mild hypocalcemia could be asymptomatic. Interestingly, the most common cause of hypocalcemia is radical resection of the head and neck due to cancer. The direct impact of hypocalcemia on teeth or craniofacial bones is largely invisible. The treatment for this disorder is vitamin D and calcium supplementation.

Hypercalcemia refers to blood calcium levels above normal, which can lead to diffuse precipitation of calcium phosphate (ectopic ossification) in tissues, leading to widespread organ dysfunction and damage. The most common cause of hypercalcemia is hyperparathyroidism (an overproduction of the parathyroid hormone), which tends to occur more in women. Other medical disorders such as kidney failure or cancer can result in severe hypercalcemia, and may require hospitalization to lower calcium levels. Again, the impact of

hypercalcemia on craniofacial bones and teeth is less noticeable.

Phosphate homeostasis and its impact on craniofacial bone and teeth

Phosphate distribution and balance

Phosphate, like calcium, is distributed in every cell and has multiple functions; it is a component of DNA and RNA and an essential molecule for energy and cell signaling. There are approximately 1000 g of phosphate in an adult, which is less than the approximate amount of calcium. Most of the phosphorus (>85%) is in hard tissues, which is different from calcium distribution (99% in hard tissues). Phosphate in nonhard tissues (~15%) exists mainly in the intracellular fluid in the form of inorganic phosphate, with only 1% present in the extracellular fluid, whereas calcium is present primarily in nonhard tissue extracellular fluid. Phosphorus is present in a wide variety of foods, whereas calcium has a more limited food presence. In most cases, therefore, dietary phosphate supply is not a nutritional issue, while calcium supplements are often recommended throughout the life span, including the period of early development of craniofacial bones and teeth. In contrast to the tightly controlled calcium levels, normal serum phosphate concentrations in adults range between 2.7 and 4.5 mg/dL (0.87–1.45 mM), with a range of approximately $\pm 25\%$ from the average concentration, and are affected greatly by diet. Furthermore, concentrations of both calcium and phosphate are age dependent, with the highest levels occurring during the neonatal period. Both phosphate and calcium levels display circadian rhythms, although each is different from the other. The peak level of phosphate occurs in the late morning, but calcium peaks in the evening. The impact of rickets due to hypophosphatemia (low phosphate) on craniofacial bones and teeth is more critical than that of hypocalcemia rickets, because the cause of the latter is primarily nutritional and can be efficiently treated with vitamin D and calcium supplements, while the cause of hypophosphatemic rickets is primarily genetic, which makes it difficult to treat.

Phosphate regulation

Like calcium regulation, phosphate balance is regulated by three resources: (1) absorption of phosphate from the intestines (~16 mg/kg body weight/day), (2) phosphate reservoir in skeleton (3 mg/kg body weight/day), and (3) phosphate excretion in urine (~13 mg/kg body weight/day) (Bergwitz & Juppner 2010). The phosphate balance is controlled by PTH and 1,25(OH) $_2$ D $_3$ (similar to

calcium balance), as well as fibroblastic growth factor 23 (FGF23) that has no direct role in calcium balance.

Phosphate regulation by PTH and 1,25(OH) $_2$ D $_3$

Although PTH functions as the key hormone for regulation of calcium homeostasis, it plays an important role in the control of phosphate homeostasis. PTH regulates phosphate in three organs: the (1) skeleton (indirectly on osteoclast cells via osteoblast cells), (2) kidney (a direct role via the proximal convoluted and proximal straight tubule), and (3) intestine (an indirect role via 1,25(OH) $_2$ D $_3$) (Bergwitz & Juppner 2010). Low calcium or high phosphate stimulates the parathyroid glands, which increases the production and release of PTH into serum. At the targeted organs, PTH binds to the PTH/PTHrP-receptor (PTHrP), a G protein-coupled receptor, followed by activations of several signaling pathways such as MAP kinase, Gs α /PKA, and Gq/PLC/PKC. In bone, osteoclast cells do not have PTHrP, but osteoblasts do. PTH directly targets osteoblasts where RANKL is released to activate osteoclast differentiation and maturation. As a result, bone resorption occurs and the levels of calcium and phosphate are increased in serum. In kidneys, PTH targets on the same receptor and increases calcium reabsorption, but it inhibits phosphate reabsorption via internalization of the sodium-phosphate co-transporters NaPi-IIa and NaPi-IIc. The net immediate short-term effect of PTH on the regulation of phosphate and calcium is an increase in serum calcium and a decrease in serum phosphate. In the long term, PTH stimulates production of CYP27B1 (25-hydroxyvitamin D $_3$ 1-alpha-hydroxylase) in the proximal tubule of the kidney. This enzyme catalyzes the 1-alpha-hydroxylation for production of 1,25 D $_3$ (the bioactive form of Vitamin D), which then increases absorption of calcium and phosphate in the intestine. Note that 1,25(OH) $_2$ D $_3$ is not the only molecule responsible for calcium and phosphate absorption in the intestine, and there are other factors that affect absorption of phosphate and calcium (Quarles 2008).

Phosphate regulation by FGF23

The discovery of FGF23, a unique member of the FGF superfamily, has clarified much of our understanding of not only abnormalities in phosphorus homeostasis, but also normal regulation of phosphate levels. This 32 kDa protein is primarily produced by osteoblasts and osteocytes in bone (Feng *et al.* 2006). Deletion of the DMP1 (dentin matrix protein) or Phex (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) gene in mice or mutations of DMP1 or PHEX in humans leads to a sharp increase in FGF23 in osteocytes (Feng *et al.* 2006; Qin *et al.* 2007; Yuan *et al.* 2008;

Bergwitz & Juppner 2010). Note that murine odontoblast cells also express FGF23, although the physiological significance is not clear (Jiang *et al.* 2010).

The kidney is the primarily physiological target for FGF23. Recent work suggests that the N-terminus of FGF23 interacts with FGFR1c, and its C-terminus interacts with klotho, a FGF23 co-receptor that is mainly expressed in the kidney. The direct consequence of the activation of these complexes is the acceleration of phosphate efflux via an inhibition of NPT2a and NPT2c and a decrease in the synthesis of $1,25(\text{OH})_2\text{D}_3$ through blocking CYP27B1 activity in the kidney. FGF23 also targets parathyroid glands to inhibit PTH expression and secretion. Taken together, FGF23 is the most critical regulator in phosphate homeostasis as opposed to PTH, which is mainly responsible for calcium homeostasis.

There are many physiological and pathological factors that regulate FGF23. Physiologically an increase in $1,25(\text{OH})_2\text{D}$ levels or dietary phosphorus intake induces FGF23 production. This forms a perfect feedback loop that keeps phosphate at a relatively stable level (i.e., FGF23-mediated phosphaturia prevents potential hyperphosphatemia from enhanced $1,25(\text{OH})_2\text{D}$ -dependent intestinal phosphate absorption). Pathologically, several local factors such as PHEX and DMP1, which increase FGF23 levels when deleted in mice or mutated in humans, have been identified (Quarles 2008).

PHEX is a cell surface endopeptidase located predominantly in odontoblasts, cementoblasts, osteoblasts, and osteocytes. Deletion of *PheX* in mice or mutations of this gene in humans leads to an increase of FGF23 in osteocytes. Deletion of *Dmp1* in mice or mutations of DMP1 in humans results in nearly identical increments of FGF23 in osteocytes, leading to hypophosphatemia, aberrant vitamin D metabolism, and rickets and osteomalacia. Ablation of FGF23 or blocking FGF23 with monoclonal antibodies against FGF23 corrects these abnormalities in both *PheX*- and *Dmp1*-deficient mice (Feng *et al.* 2006; Quarles 2008). Interestingly, overexpression of the full length of DMP1 or its 57kDa C-terminus in normal osteoblast and osteocyte cells has no direct role on FGF23 expression, but the targeted expression of full-length DMP1 or the C-terminus in *Dmp1*-null osteoblast and osteocyte cells fully restores FGF23 levels and rescues *Dmp1*-null phenotype, including growth plate defects, osteomalacia, abnormal osteocyte maturation, and the abnormal osteocyte lacunocanalicular system, as well as hypophosphatemia (Lu *et al.* 2011). These results support the hypothesis that gene expression levels are reduced during maturation of osteoblasts into osteocytes, and DMP1 is one of the key molecules that controls this downsizing process. A loss of DMP1 will disrupt normal osteoblast-to-osteocyte

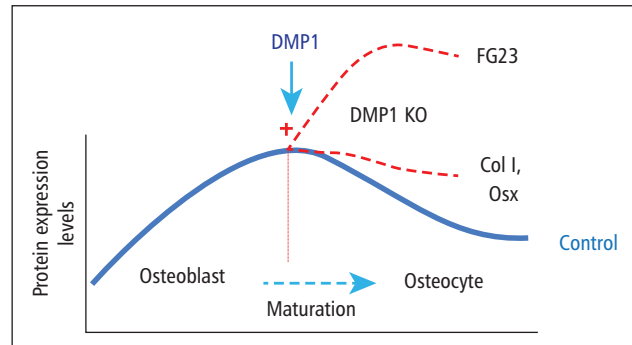


Figure 34.1 Regulation of osteocyte maturation by DMP1. Protein expression levels are high in matrix-producing osteoblasts, whereas protein expression levels are reduced during osteocyte maturation. DMP1 is one of the key players during this process. A loss or mutation of DMP1-KO will disrupt maturation of osteoblasts into osteocytes. As a result some genes, such as FGF23 normally produced in osteoblasts, are ectopically overproduced in the *Dmp1*-KO osteocytes (Feng *et al.* 2006). Some genes, such as type I collagen (Col I) or osterix (Osx) that are mainly produced in normal osteoblasts with only a small amount produced in osteocytes, are abnormally elevated in embedded *Dmp1*-KO osteocytes. This may explain why FGF23 is ectopically maintained at high levels in the *Dmp1*-KO osteocytes (unique roles of phosphorus in endochondral bone formation and osteocyte maturation, 10.1002/jbmr.294).

maturation, and the expression of many genes, such as FGF23, is abnormally elevated in embedded osteocytes (Figure 34.1).

Impact of the disturbance of phosphate homeostasis on craniofacial bones and teeth

Any cause that changes the phosphate balance (either lowering it or raising it) will lead to health problems in humans. For example, hyperphosphatemia commonly occurs in individuals with renal insufficiency. To some degree, most patients with acute or chronic renal failure have hyperphosphatemia. Although acute hyperphosphatemia itself is not a serious health problem, it can lead to hypocalcemia, which is life threatening, via precipitating calcium, decreasing vitamin D production, and interfering with PTH bone resorption. Prolonged hyperphosphatemia will cause the following severe medical problems: an abnormal deposition of calcium phosphate in cardiac valves and excess free phosphate taken into vascular smooth muscle via a sodium-phosphate co-transporter. The increased cellular phosphate induces Runx2, which stimulates ectopic ossification in vascular walls and leads to increased systolic blood pressure and subsequent left ventricular hypertrophy.

However, the most problematic disturbance of phosphate homeostasis that affects craniofacial bones and

teeth is hypophosphatemic rickets. Unlike calcium, hypophosphatemia is mainly induced by a genetic defect, not by nutritional deficiency. Here we focus on two genes whose deletion or mutations lead to pathological changes in craniofacial bones and teeth partly due to hypophosphatemia.

X-linked hypophosphatemia (XLH) is a heritable type of vitamin-D resistant rickets that is associated with PHEX mutations and accounts for most of the reported cases of hypophosphatemia rickets. Because PHEX is located on the X chromosome and males have only one X chromosome, this disease is highly expressive in males. Biochemically, XLH is recognized by hypophosphatemia and inappropriately low levels of 1,25-OH vitamin D₃, which are a consequence of high FGF23 levels. Clinically, the most common bone phenotype is short stature and bowed legs. The tooth phenotype is characterized by interglobular dentin, widened predentin, and irregular dentinal tubules. Patients with severe XLH exhibit enamel defects. The Hyp mouse, which is caused by the *PheX* deletion mutation, is a murine homolog of human XLH and is widely used for human XLH rickets studies. The Hyp mice display a similar phenotype to that of XLH except that the increase in FGF23 is more pronounced and cementum defects have been identified (Yuan *et al.* 2008; Fong *et al.* 2009).

DMP1 mutations in autosomal recessive hypophosphatemic rickets (ARHR) patients and mice lacking DMP1 display an overlapping pathophysiology such as hypophosphatemia. However, subtle differences exist between the mouse model and human ARHR patients. For example, FGF23 levels are extremely high in *Dmp1*-KO mice but only slightly elevated in human patients (Feng *et al.* 2006). Second, a defect in bone remodeling is very severe in *Dmp1*-null mice, as shown by a greater accumulation of trabecular bone in the bone marrow space and expanded metaphyses plus the development of large bony protuberances. Mechanism studies show a sharp reduction of osteoclast numbers and RANKL expression in combination with three times more OPG in the *Dmp1*-KO mouse long bone. These defects are caused in part by hypophosphatemia, since injection of monoclonal antibodies against FGF23 greatly improves the above phenotype (Figure 34.2; Zhang *et al.* 2011). These differences are partly due to the fact that the mutant DMP1 remains part of the DMP1 function (Jiang *et al.* 2010) and partly due to species specificity of human versus mouse.

Conclusion and future directions

For many years, biomedical researchers focused on calcium homeostasis because (1) disturbances of calcium

homeostasis cause significant medical problems, especially in children and senior populations; and (2) phosphate, unlike calcium, is present in almost all foods and thus nutritional deficiencies are not an issue in most cases. In the last 10 years, remarkable progress has been made in understanding phosphate homeostasis by focusing on genetic disorders associated with phosphate regulation and linking this with genetically engineered mouse models. The discovery of FGF23, the key hormone for regulation of phosphate homeostasis, has stimulated interest in understanding the causes for disorders of phosphate homeostasis in craniofacial bones and teeth, as well as in renal physiology. However, many challenging questions remain to be answered. For example, we do not know the detailed mechanisms by which dietary intake and serum phosphate levels negatively regulate PTH, FGF23, and 1,25(OH)₂D₃. Largely unknown is how loss-of-function DMP1 and PHEX results in an increase in FGF23 gene transcription. Although it is clear that FGF23, Klotho (co-receptor), and FGF receptors form complexes that inhibit phosphate reabsorption via NPT2 co-transporters, the mystery is that Klotho and FGF receptors do not locate at the same renal tubule cells (how can they move together to form these complexes?). Another perplexing puzzle is that an increase in FGF23 is the primary cause of hypophosphatemia rickets in all animal models, but an increase of FGF23 is not linked with all cases of hypophosphatemia rickets. By continuing to elucidate these issues, we will gain new insights into the key regulators required for normal function of bones and teeth throughout the life span.

Summary

Mineral metabolism is essential for proper cell functions, and for the formation of craniofacial bones and teeth. Almost all tissue calcium (99%) is located in hard tissues with only 1% located in extracellular fluid. Most phosphate (~85%) is present in hard tissues with 15% distributed in soft tissues. In contrast to tightly controlled calcium concentration, phosphate concentration has an approximate 25% deviation from the average concentration. Calcium and phosphate homeostasis is controlled by parathyroid hormone (PTH), 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), and fibroblast growth factor 23 (FGF23, a more recently discovered hormone). In the intestine, 1,25(OH)₂D₃ promotes calcium and phosphate absorption. In bone, PTH increases bone resorption to release calcium and phosphate. In the kidney, PTH enhances calcium reabsorption and increases excretion of phosphate. FGF23, which is primarily released from bone cells, specifically accelerates phosphate excretion

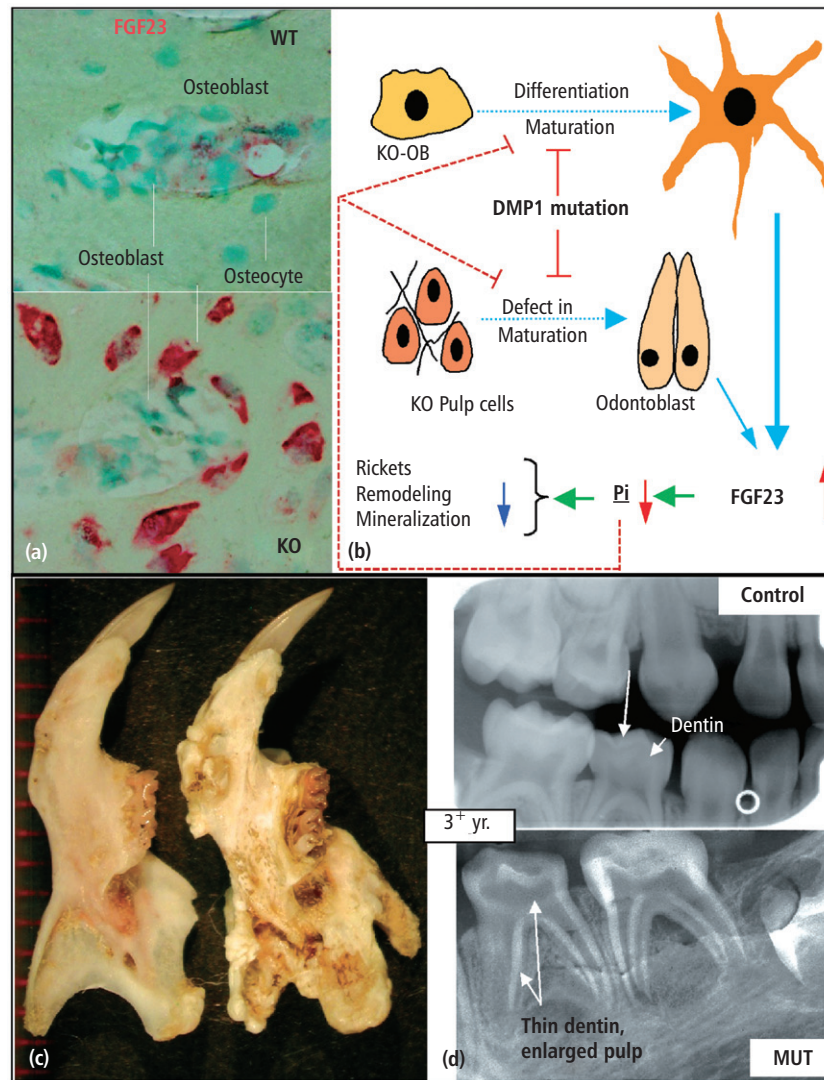


Figure 34.2 Impact of DMP1 mutations on craniofacial bones and teeth. 1. In situ hybridization shows a sharp increase in FGF23 mRNA (red) from 10-day-old *Dmp1*-KO osteocytes obtained from jaws (upper panel: control; lower panel: KO). (The data are adapted from Feng *et al.* 2006.) 2. Summary of defects of Pi homeostasis in *Dmp1*-KO mice. Note that FGF23 is mainly released from normal osteoblast and odontoblast cells that target kidneys for inhibition of Pi reabsorption. In pathological conditions such as mutation or deletion of DMP1, overproduction of FGF23 in bones and teeth leads to hypophosphatemia rickets, and defects of remodeling and mineralization. 3. A severe defect in a 1-year-old *Dmp1*-KO jaw. (Data are adapted from Zhang *et al.* 2011.) 4. A 3-year-old ARHR patient displays severe reduction in dentin thickness, large pulp/root canals, and a change in tooth shape (lower panel). (Data are adapted from Jiang *et al.* 2010.)

and inhibits $1,25(\text{OH})_2\text{D}_3$ production in kidneys. Any significant change of calcium and phosphate homeostasis will lead to disease. Disturbances of calcium and phosphate homeostasis due to gene mutations greatly change bone and tooth structure and function. Two hypophosphatemia rickets forms caused by mutations of PHEX and DMP1 are described in this chapter.

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Clinical correlate: mineral metabolism and disruption of dentoalveolar development in a case of craniometaphyseal dysplasia (CMD)

Hai Zhang and Brian Foster

Craniometaphyseal dysplasia (CMD) is a rare congenital disorder characterized by marked sclerosis and increased density of the craniofacial bones (MIM 123000). Narrowing of the cranial foramina can result in secondary pathologies including facial paralysis or palsy, blindness, deafness, and neurological impairment. Metaphyses of long bones are widened, but the extracranial skeleton and joints are otherwise unaffected or very mildly affected.

Autosomal dominant CMD has been linked to mutations in *Ankh*, the gene for the progressive ankylosis protein (Nürnberg *et al.* 2001; Reichenberger *et al.* 2001; Kornak *et al.* 2010). *ANKH* is a membrane protein that regulates transport of pyrophosphate (PP_i), a potent inhibitor of both physiologic and pathologic mineralization (Ho *et al.* 2000). Loss of *ANKH* function has been proposed as the mechanism for bone pathologies observed in CMD, wherein the resulting extracellular PP_i deficiency would contribute to underregulated mineral tissue formation (Gurley *et al.* 2006b). Interestingly, when the homologous gene *Ank* harbors a substitution mutation or is knocked out by targeted deletion in mice, the resulting phenotype features severe progressive arthritis of the joints, but no craniofacial bone phenotype (Gurley *et al.* 2006a). In both *Ank* mutant and knockout mice, we identified a dramatic tooth phenotype with significantly expanded acellular cementum of the root (Nociti *et al.* 2002; Foster *et al.* 2011).

In addition to the hallmark craniofacial features for CMD, reports have noted tooth abnormalities including malocclusion, crowding, and delayed eruption, though

detailed dental examinations have not been published (Hayashibara *et al.* 2000; Lamazza *et al.* 2009; Kornak *et al.* 2010). The purpose of this case report is to describe both systemic and dental manifestations in a patient with CMD. This may help inform both physicians and pediatric dentists who encounter cases of CMD in their practices. The potential genetic and molecular mechanisms of how *Ankh* mutations affect tooth root development are discussed in relation to CMD and other conditions.

Case presentation

Past medical history

The male patient was born to a 29-year-old, gravida 1, para 0–1 mother at 39 weeks gestation via normal spontaneous vaginal delivery and weighed 3.4 kg (7.5 lbs). Soon after birth, the patient's family noticed a difference in his facial appearance. At approximately six months of age, he was evaluated in a local emergency room for concerns related to upper airway compromise. At that time, the family was informed that the child had a "genetic disorder." Subsequent evaluations led to the diagnosis of CMD. The patient experienced chronic nasal congestion, excessive snoring, a mild nasal discharge, and mouth breathing with upper respiratory tract infections, but no other medical problems.

Family history

Family history was negative for any skeletal or dental anomalies. At the time the patient was seen, a younger

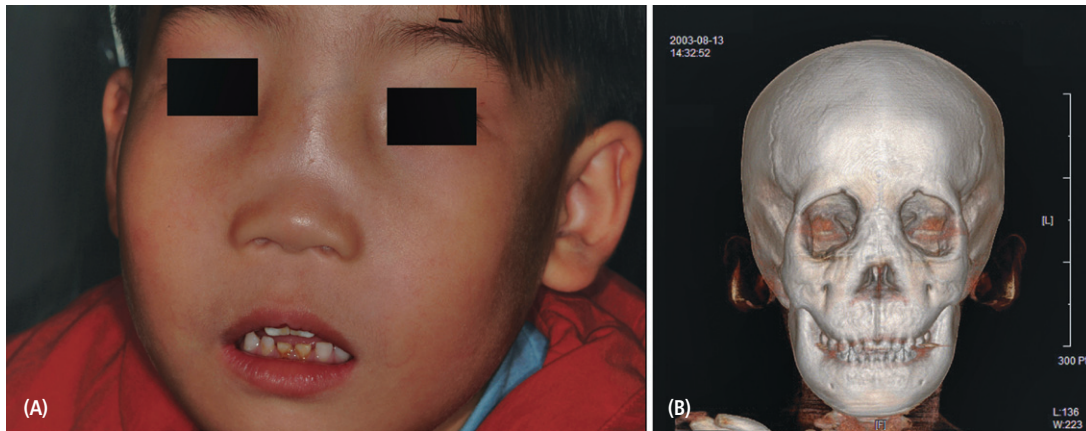


Figure 35.1 A facial photo (A) and a three-dimensional (3D) CT scan image (B) of the skull, both demonstrating the classic facial features of craniometaphysal dysplasia with dramatic thickening of the nasal bones and the nasal glabellar region.

and apparently healthy two-month-old baby girl had been born into the family. Neither his parents nor his sister have features of CMD, which suggests that this case represents a spontaneous mutation.

Physical examination

At 3.5 years of age, the young boy was referred to the Children's Craniofacial Center, Children's Hospital and Medical Center, Seattle, Washington, for evaluation. Height, weight, and occipital frontal circumference all fell between the 75th and 90th percentiles. He had the classic facial features of an autosomal dominant CMD, with dramatic thickening of the nasal bones and the nasal glabellar region resulting in leonine facies (Figure 35.1). Nasal endoscopy revealed normal nostrils but severe intranasal stenosis from medialization of the turbinates. The patient had significant thickening of the zygoma, the zygomatic arches, and particularly the mandibular ramus and body. He had the appearance of hypertelorism due to lateral displacement of the lateral canthi and the breadth of his nose. Intracanthal distance (37 mm) and the interpupillary distance (53 mm) were normal. His visual acuity was 20/25 on the right and 20/20 on the left by Allen picture testing. Cranial nerves II–XII were intact, and fundoscopic examination showed no evidence of papilledema or nystagmus. His tone and reflexes were symmetric. The external ears were normally formed. Oropharyngeal examination revealed broadening of the alveoli, but no overt mucosal disease or palatal malformations otherwise. His axial and appendicular skeleton was normal with no evidence of metaphysal flaring. The remainder of his general physical exam revealed nothing significant.

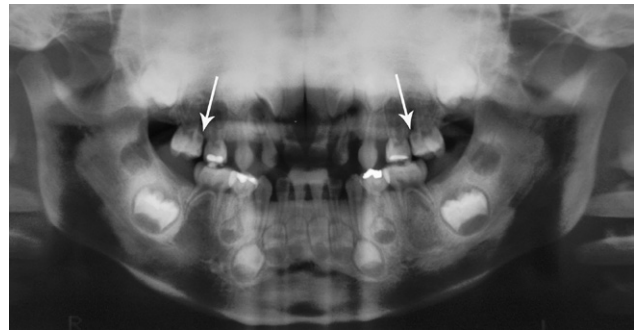


Figure 35.2 A panoramic radiograph of the patient's orofacial tissues. Thick bones were present in the midfacial area. The radiographic appearance of all primary teeth as well as the development of the permanent dentition appeared to be normal. The arrows indicate the "enamel pearl"—like excess mineralization of the primary maxillary second molars.

Radiographic findings

Computed tomography (CT) scans revealed features of CMD, with dramatic thickening of all of the facial bones: vomer, nasal turbinates, medial pterygoids, middle ear ossicles, calvaria, skull base, and narrowed optic foramina (Figure 35.1B). Neither CT nor magnetic resonance imaging (MRI) scans demonstrated evidence of ventriculomegaly or increased intracranial pressure. Panoramic radiographs revealed anomalies of the developing permanent dentition (Figure 35.2). In the maxillary arch, the permanent maxillary central incisors (teeth 8 and 9 in the Universal Numbering System) were abnormally shaped and rotated. Developing tooth buds for the remaining permanent teeth were observed, except for the mandibular second premolars (20 and 29). The tooth

buds superimposed over the maxillary lateral incisors (7 and 10) might be supernumerary teeth, although we were not able to confirm this because maxillary occlusal radiographs were not available.

Oral examination

Upon presentation for clinical oral examination, the soft tissues, dentition, and occlusion were examined. The intraoral soft tissues were within normal limits. The marginal gingiva was mildly inflamed, which was consistent with gingivitis due to local factors. A full complement of 20 primary teeth in occlusion was noted. The sizes of all of the teeth were within the normal clinical range. Thus, the radiographic appearance of macrodontia molars is likely due to distortion of the panoramic radiograph. Primary canines exhibited a Class I relationship. Overbite was 20%, and overjet was 3 mm. The patient had a telescopic bite with the mandibular transverse dimension narrower than the maxillary transverse dimension and, upon occluding, his mandibular posterior teeth were palatal to the maxillary posterior teeth. In orthodontic terminology, this is known as a *Brodie bite*. This may result from the excessive thickness of maxillary bone, which in turn secondarily affects dental occlusal relationships. The thickness of the bone will complicate future treatment approaches. There was no evidence of active dental caries, although some restorative treatment had previously been performed on four primary teeth. Many of the teeth exhibited dysmorphic and discolored surfaces. This was particularly striking in the primary mandibular central incisors, O and P (Figure 35.3), and the primary maxillary first molars, B and I (Figure 35.4). The labial surface of the primary mandibular central incisors exhibited a brownish-orange discoloration consistent with enamel dysplasia and/or enamel hypoplasia. The primary maxillary second molars showed a similar malformation, with the addition of what appeared to be an enamel pearl or similar feature on the buccal surface near the mesiobuccal line angle bilaterally (Figure 35.2). Rubber cup pumice prophylaxis did not affect any of the visual findings other than the removal of plaque.

Clinical follow-up

While several dentoalveolar anomalies of the primary teeth and developing permanent dentition were noted, the dental phenotype in this case did not warrant management at the time of observation. Additional clinical exams and patient care over the long term were prevented by the family relocating. Although no standard therapy for the extensive thickening of the craniofacial skeleton exists, debulking procedures to decompress

increased intracranial pressure, entrapped cranial nerves, and compression of the brain stem are frequently temporizing (Day *et al.* 1997; Puri & Chan 2003; Sheppard *et al.* 2003; Ahmad *et al.* 2006).

Discussion

Functional properties of the dentoalveolar complex depend on calcium (Ca^{2+}) and phosphate (P_i) metabolism, which constitute the building blocks for hydroxyapatite (HAP). While the dynamic regulation of Ca^{2+} has been understood for some time, only in the last decade has it become recognized that the P_i metabolism is subject to active and complex feedback among multiple organ systems, including the digestive system, kidneys,



Figure 35.3 A frontal view of the upper and lower anterior teeth showing discoloration of the tooth surfaces. The arrow indicates the malformation or excess mineralization of the mandibular central incisor.



Figure 35.4 A lateral view of the primary maxillary right first molar showing the discoloration and defects on the tooth surface.

parathyroid glands, and bones and teeth. (For a review, see Chapter 16 in this volume, and Foster *et al.* 2008). Systemic P_i is regulated by modulation of dietary absorption, renal reabsorption, and mobilization from bone. However, mineralization is also controlled at the local tissue and cell levels, directed by a multitude of mineral-binding extracellular matrix proteins, proteoglycans, and regulators and inhibitors of mineralization. One such is pyrophosphate (PP_i), an inhibitor of physiologic and pathologic mineral (Murshed *et al.* 2005). Modulation of PP_i concentration at sites of bone and tooth mineralization and removal of PP_i by hydrolysis are thus important processes guiding proper hard-tissue formation. The progressive ankylosis protein (ANK) has been identified as one of the key cellular regulators of PP_i , and it functions by controlling transport of PP_i from intracellular to extracellular compartments (Ho *et al.* 2000; Gurley *et al.* 2006a, 2006b). Along with other factors, including ectonucleotide pyrophosphatase phosphodiesterase 1 (NPP1) and tissue nonspecific alkaline phosphatase (TNAP), ANK governs local concentrations of PP_i , dictating the ratio of P_i to PP_i and mineralizing conditions. Mutations in the *Ankh* gene, located on chromosome 5, cause autosomal dominant craniofacial dysplasia (CMD), a developmental disorder featuring sclerosis and increased density of the craniofacial bones, with only mild effects on the extracranial skeleton and joints (MIM 123000; Nürnberg *et al.* 2001; Reichenberger *et al.* 2001; Kornak *et al.* 2010). While transmission of CMD phenotypic features is reported to be complete, the severity of expressivity is variable in the case report literature. The patient in this case presented classic craniofacial features of CMD, namely, wide nasal bridge and paranasal bossing, hypertelorism, and thickening of the zygoma, zygomatic arches, and mandible. His extracranial skeleton was normal and showed no evidence of metaphyseal flaring at the time of observation.

One of the goals of this study was to describe the dental phenotype in the context of the larger craniofacial manifestation of CMD. One reason for this is that ANKH has been implicated in tooth root development in murine models of *Ank* loss of function. In both *Ank* mutant and knockout mice, the cervical cementum of the tooth root was significantly expanded compared to controls, while other dentoalveolar tissues were unaffected (Nociti *et al.* 2002; Foster *et al.* 2011). In the case examined here, the enamel discoloration and malformation were consistent with enamel hypoplasia or dysplasia. There are many potential causes for these enamel conditions, but their presence could point toward a previously undescribed role for P_i and PP_i in amelogenesis or enamel mineralization. Characteristic hyperostosis and sclerosis of man-

dibular bone led to malocclusion in this patient, and the bone phenotype generally made it difficult to assess radiographically some aspects of tooth root appearance, especially in the maxilla. In light of the mouse hypercementosis phenotype resulting from loss of ANK function, the observation of excess mineralization on maxillary primary second molars becomes very interesting. While consistent with “enamel pearl” formation, the extent of this excess mineralization is actually unclear, as is its relation to the roots. Ideally the patient would have been monitored over time to manage the craniofacial aspects of CMD, and tooth development could have been followed, allowing for evaluation of the eruption and morphology of permanent dentition, and even a chance for histological examination of deciduous teeth after exfoliation. Unfortunately, the patient was removed from our care when the family moved out of the area.

While the *Ank* gene is highly conserved across vertebrates, unexplained inconsistencies between human mutations and mouse models persist, and so several key aspects of the ANK function remain unclear at present. Mutations linked to CMD tend to be point mutations, clustering in putative cytoplasmic domains near the C-terminus of ANKH. However, mutations in the N-terminus cause familial chondrocalcinosis (MIM 118600, CCAL2), also called calcium pyrophosphate interact deposition disease (CPPDD; Pendleton *et al.* 2002). CCAL2 patients feature pathological cartilage and articular calcification as a result of excess PP_i in these tissues, which leads to the proposal that these are gain-of-function mutations, though this remains controversial (Gurley *et al.* 2006b). A critical clue for the mechanism of CMD was supplied by Chen and colleagues (2009) wherein a knock-in mouse was produced expressing a human mutation (Phe377 deletion) associated with CMD (Chen *et al.* 2009). Like human CMD, the *Ank* homozygous knock-in mice featured craniofacial and mandibular hyperostosis, decreased foramina, obliteration of nasal sinuses, and long-bone defects, with a milder phenotype in heterozygotes. The CMD-like pathology seems to be related to increased bone turnover and reduced osteoclastogenesis, which especially target craniofacial bone, and it is hypothesized that mutations in *Ankh* can affect protein–protein interactions that remain unidentified at present, in addition to affecting PP_i transport. A second important clue came with identification of an autosomal recessive disorder featuring progressive ankylosis of joints, as well as other pathologies including neurological symptoms and osteopenia in the presence of mild hyperphosphatemia, for which etiology was identified as an L244S mutation in *Ankh* (Morava *et al.* 2010). This finding was important because it is the first recessive disorder identified for *Ankh* in

humans and because of the joint pathology, both of which validate the homology of function for mouse and human ANK proteins. Dental anomalies were noted but have not yet been described in detail. This all supports a potential role for ANKH in human odontogenesis.

Conclusion

Findings reported here underscore the need to characterize the oral–tooth phenotype in disorders linked with craniofacial anomalies. Continued characterization of the orodental phenotype observed in patients with genetic disorders of phosphate and PP_i metabolism will assist in understanding the role of these factors in modulating mineral formation, as well as providing strategies for improving therapies targeted at controlling abnormal mineral metabolism disorders.

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Sun, nutrition, and the mineralization of bones and teeth

Philippe P. Hujoel

Sunbaths are part of the regimen of every well-cared-for infant.

—Palmer and Alpher (1937)

Niels Finsen became the only dermatologist to receive the Nobel Prize in Physiology or Medicine in 1903 for the medical applications of ultraviolet (UV) radiation. Heliotherapy, the therapeutic use of sunlight or lamps with specific wavelengths, became popular in the early twentieth century and was hypothesized to have many benefits, including the mineralization of teeth and bones. Sunbaths became part of a landmark US public health intervention for improving bone health in 1923 (Eliot 1925).

The turn of the twenty-first century sounded a death knell for the natural sources of vitamin D in our lives. Beliefs on the health benefits of sunlight and animal fats—the two primary vitamin D sources—came into question. Heliotherapy became a thing of the past when august institutions such as the World Health Organization (WHO) recommended that infants always remain in the shade (WHO 2006). Why shut down the sunlight-activated endocrine vitamin D system when it is most in demand by the dental and skeletal tissues? The rationale rested on at least two assumptions: effective skin cancer prevention and the equivalence of dietary vitamin D supplements to sunshine (Wagner & Greer 2008).

Beliefs on the health benefits of sunlight were not the only medical recommendations to change during the twentieth century; views surrounding other sources of vitamin D experienced similar, though likely unrelated,

changes during the same time period. Animal fats, a source of vitamin D, switched from being a desirable nutrient to a nutritional taboo. In contrast, sugar and other refined carbohydrates went from being nutritional bane to desirable health snacks. These late-twentieth-century dietary beliefs are illustrated, for instance, in the National Cholesterol Education Program which describes hard candy, angel cake, and jelly beans as heart-healthy snacks and daily egg consumption as harmful (National Heart, Lung, and Blood Institute, National Institutes of Health 2002). Given that sugary “heart-healthy” snacks cause dental caries, fluoride joined vitamin D as a necessary drug for maintaining health (Palmer & Wolfe 2005).

There are no pivotal clinical trials available to tell us whether avoiding sun and animal fats improved either dental or overall health. This chapter will review lesser evidence to explore the current health advice on sun and nutrition within a historical context. The nineteenth century, in the middle of an epidemic in which toddler bones were so weak that they bent under the strain of walking or chewing, may be a good place to start this story.

Rickets and industrial foods: the pre-vitamin, pre-World War I era

Vegetable oils were useless in curing human rickets (Chick 1975; Davenport-Hines & Slinn 1992), a statement attributed to Armand Trousseau (1801–1867)—a French physician who was the first to describe hemochromatosis, first to perform a tracheotomy, first to aspirate the pleural cavity, and author of several classical medical textbooks, one of which described fish oil as the

cure for rickets and pap and poor climate as causing rickets (Dunn 1999). Vegetable oils were used in the 1920s to induce experimental rickets. In the 1970s, these vegetable oils became advocated for heart health. The American Heart Association (AHA) still recommended vegetable oils in 2009 (Harris *et al.* 2009), even though in randomized trials they do not appear to reduce mortality risk (Ramsden *et al.* 2010).

Rickets is a disease of infancy and early childhood characterized by delayed eruption of teeth, delayed walking, beading of joints, and bending of bones when stressed. It has been suggested that even the mandible may bend under the strain of occlusal forces (Figure 36.1).

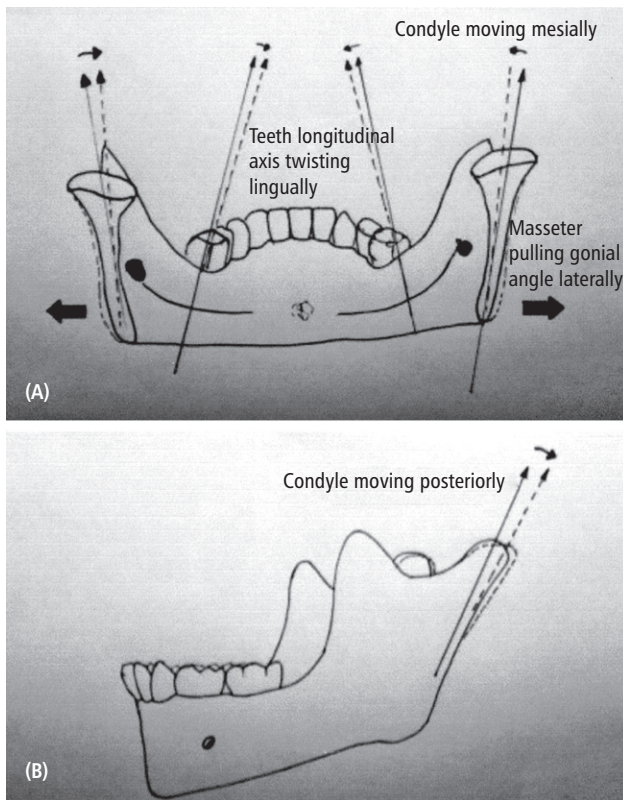


Figure 36.1 Posterior and lateral view of the mandible of a three-year-old child. Muscle forces can move the condyle head mesially (A) and posteriorly (B) when the bone is poorly mineralized and malleable. These same forces also cause occlusal forces that twist the lower molars lingually and crowd the incisors. The plausibility of this scenario is supported by anthropologists who reported the condyle head moving medially and posteriorly with rickets (Mays 2002). Others have argued, however, that since mandibles do not have an epiphysis, no changes in bone structure are to be expected (Hatfield 1910). Dental crowding so often noticed in rickety rats or dogs needs to be extrapolated to humans with caution. These animals, in contrast to humans, do have a patent mandibular epiphysis.

Common symptoms include an unusual skull shape with parietal and frontal bossing, knock knees or bowlegs, and concave chest walls. Infants or children who suffer from such symptoms are referred to as *rachitic*, and interventions that improve rickets symptoms are referred to as *anti-rachitic*.

Rickets, like dental cavities, made its entry onto the world stage after the Agricultural Revolution (Mays 2002) and the rise in cereal consumption. It was, for instance, described in Roman times in the first and second centuries CE (Rajakumar 2003). Rickets was mistakenly described as a new disease in the mid-1600s when refined sugar, another carbohydrate, began its climb in popularity. It was initially reported to be more common among the wealthy. Some English royal family members, such as James VI (1566–1625) and Princess Elizabeth (1635–1650), suffered from rickets. However, the occurrence of the disease surged among the poor during the Industrial Revolution. In England, there was a 10-fold increase in rickets between the medieval times (tenth to sixteenth centuries) and the early Industrial Revolution (eighteenth and nineteenth centuries; Mays 2002). The rickets epidemic peaked in the late nineteenth to early twentieth centuries, after which it again became rare. The disease was often endemic (McCollum 1922). Great Britain, Iceland, and the Isle of Lewis, all islands at high northern latitudes, had widely varying rickets rates (McCollum 1922). Within Great Britain, there was a high prevalence in London and the South West and a low prevalence in the North (Dunnigan 2003).

There were two suspected causes: a lack of sunshine and increased consumption of processed foods. A lack of sunshine in the northern cities was a suspected cause of rickets in the late 1800s (Figure 36.2). Palm (1890) deduced as much by documenting the absence of rickets in the tropics. With the discovery of UV therapy, split-body experiments would demonstrate that UV light on one side of the body cured rickets symptoms on both sides of the body, suggesting that local skin exposures had systemic anti-rachitic effects (Huldschinsky 1928). Sun exposure explained why hunter-gatherers escaped the disease and why poor women of the Industrial Revolution toiling away indoors had rachitic infants. However, the sunshine hypothesis did not explain why primitive Eskimos escaped the disease.

Processed foods were also recognized as playing a detrimental and independent role in the health of bones and teeth. Populations isolated from industrial foods were free from rickets, and a switch to refined foods was associated with the appearance of rickets (McCollum 1922; Price 1940). Proprietary infant formulas poor in fats and high in sugar were held responsible for rickets

and “a holocaust of infants” (Davenport-Hines & Slinn 1992). The growth in the infant formula industry in the late nineteenth century appeared to coincide with a surge in the occurrence of rickets. Cereals were recognized as another driver of the rickets epidemic as early as 1866, with suspected causal components being the pure carbohydrates, the phytates that complemented calcium, or the industrial additives that complemented phosphates. John Snow, an icon in infectious disease epidemiology, hypothesized that alum, an aluminum-containing compound used to whiten bread, caused rickets (Dunnigan 2003). Aluminum was later shown to induce rickets in adults (or osteomalacia), suggesting that there was merit to his hypothesis (Bloom & Flinchum 1960; Dunnigan 2003).

Successful treatments for rickets were similarly commonly reported in the nineteenth century. In the 1820s, German physicians (Guy 1923) reported that cod liver oil cured rickets. Trousseau followed suit and advised that fish oil would cure rickets (Dunn 1999). An 1886 *Lancet* advertisement—dressed up as a nutrition review—reported the cod liver oil cure for rickets (Burroughs 1886). Even rachitic lion cubs received cod liver oil in the London Zoo in 1889 (Chesney & Hedberg 2010).

The fish oil cure led to the hypothesis that rickets was a nutritional deficiency syndrome caused by the absence of an essential nutrient. English physician George Budd, later referred to as “Prophet Budd,” suggested in 1840 that rickets, scurvy, and pellagra were nutritional deficiency syndromes (Hughes 1973); Hopkins (1906) restated this hypothesis in 1906. In addition, Funk, who coined the word *vitamine* in 1912, also suggested that rickets was a possible nutritional deficiency syndrome, as did a doctor from Johns Hopkins University in one of his recurring newspaper columns (Hirshberg 1905, 1916a; 1916b).

The possibility that rickets was a nutritional deficiency syndrome was music to the ears of industrial food companies who saw an enormous market advantage. Nutritional deficiency syndromes have a brutal logic about them: the addition of one essential nutrient can reverse epidemics of morbidity or mortality *regardless* of food processing. Scurvy, a deadly hemorrhagic condition endemic among sailors, can be cured by adding vitamin C to processed foods. Beriberi, a neurological disease endemic in certain parts of Asia, can be cured by adding vitamin B₁ to processed rice. Pellagra, a deadly disease endemic in some major maize-cultivating areas, can be cured by adding vitamin B₃ to processed corn. If rickets could be classified as a nutritional deficiency syndrome, a simple cure for another potentially harmful effect of industrial processed foods was within reach. But was

rickets a nutritional deficiency syndrome? The answer to this question turned out not to be that straightforward.

The 1920s: rickets is not a typical nutritional deficiency syndrome?

There was practically no indication from the beri-beri and the scurvy researches that the presence of any element in the diet could be considered as having a disease producing effect. . . . The results to be described will make it clear that rickets cannot be classed as a deficiency disease in this sense of the word. (Mellanby 1921)

Sir Edward Mellanby (1884–1955), a British physician and pharmacologist whose work led to the identification of vitamin D, provided experimental evidence that cereals and glucose caused rickets. This hypothesis, though confirmed and not refuted, largely disappeared from the medical literature most likely because it conflicted with the public health advice that glucose and cereals were heart-healthy.

No less than 14 vitamins, including vitamin D, were discovered between 1910 and 1946. Mellanby demonstrated that rickets-like symptoms can be induced in puppies with an experimental diet that appears indistinguishable from the cholesterol-lowering diet prescribed by the American Heart Association: bread *ad libitum* (without butter, of course), skim milk, orange juice, yeast, and vegetable fat. In 1921, he concluded that fat-soluble factor A or a factor with similar chemical properties (Mellanby 1921) needed to be added to this rachitic diet to cure rickets. McCollum, a Yale-trained biochemist, published a year later the experimental evidence demonstrating that the anti-rachitic factor was distinct from fat-soluble factor A. Vitamin D was born and named as such in 1925 (McCollum *et al.* 1922a). In 1928, the first Nobel Prize award mentioning the word “vitamin” was awarded to Windaus in part for his work on vitamin D (Wolf 2004).

Mellanby was hesitant to describe rickets as a nutritional deficiency syndrome. According to his results in dog experiments, vitamin D requirements were dependent on the unity of the diet, with cereals increasing the vitamin D requirements. Bread was a “most important substance in actually making the condition worse” (Mellanby 1921). Substituting bread with glucose led to similar findings: the more glucose, the more rickets. He suggested that carbohydrates induced obesity and that the fat produced a more urgent call on the calcium than the bone. His conclusion regarding the “pure carbohydrate moiety” left little doubt regarding his personal beliefs: “In view of the large part bread plays in the dietary of the people, the very bad effect of excessive

bread on the general health of the animals . . . cannot be too strongly emphasized” (Mellanby 1921).

His experimental animal evidence confirmed the expert opinions of clinicians of the nineteenth and early twentieth centuries, the observations of medical explorers on the etiology of rickets (Cheadle & 1906), and the opinion of industrial food companies. The principal medical officer in the United Kingdom cautioned that “the most objectionable feature” in infant formulas was the “excess of sugar” (Davenport-Hines & Slinn 1992). Quaker Oats was first to fortify oats with vitamin D, hoping that it would serve as an antidote against the cariogenic and rachitic effects of their cereals.

The role of cereals and sugar in the etiology of rickets and Mellanby’s experimental animal evidence became largely forgotten in the ensuing decades. Emphasis shifted from eliminating causes to selling antidotes. Products from beer to candy became vitamin D fortified. The extent to which food fortification or enrichment helped in eradicating the rickets epidemic is difficult to assess. Heliotherapy, use of cod liver oil, and consumption of animal fats became popular in the early 1900s and preceded or coincided with the appearance of vitamin D–fortified foods in the marketplace. For instance, in 1923 Martha Eliot, the first female elected president of the American Public Health Association and appointed chief of the Children’s Bureau by the president of the United States, started “a three-year prospective study to discover whether a supervised regimen of cod-liver oil and sunbaths could prevent rickets.” The positive results of this study were called a landmark in the field of rickets prevention (Ware & Braukman 2004). In addition, the advice given by the League of Nations (a precursor to the United Nations) not to give infants less than six months old cereal or the advice given by the Federal Children’s Bureau (currently part of the US Department of Health and Human Services) not to give a child younger than four years old ready-to-eat cereals may have helped to eliminate a cause of rickets (Palmer & Alpher 1937).

Current evidence is consistent with Mellanby’s hypotheses that vitamin D requirements depend on the totality of the diet and that carbohydrate intake increases the need for vitamin D. Chocolate and candy, the glucose intake referred to by Mellanby, have been associated with osteoporosis (Tucker *et al.* 2002; Hodgson *et al.* 2008). The US Preventive Services Task Force (USPSTF) rated the evidence of a low-serum vitamin D deficiency as a cause for rickets as fair (not good) and concluded that no diagnostic reference levels for vitamin D could be established. For pregnant women, reviewers from the *Cochrane Collaboration* concluded that there was not enough evidence to evaluate vitamin D supplementation

(Mahomed & Gülmezoglu 2009). For infants, the USPSTF concluded that bone mineral is inconsistently associated with serum vitamin D levels in infants (Cranney *et al.* 2007). For children, a *Cochrane Collaboration* review of six randomized trials concluded that vitamin D supplementation did *not* improve bone density in healthy children (Winzenberg *et al.* 2010). For adults, vitamin D supplementation alone (without calcium) is unrelated to fracture risk (Abrahamsen *et al.* 2010). While there are caveats associated with extrapolating current evidence to an almost century-old rickets epidemic, Mellanby’s conclusion that bone health requires more than vitamin D supplements remains consistent with current scientific evidence.

Vitamins and the birth of nutritionism between World Wars I and II

“Dear Old GRANDMA Meant Well . . . BUT Had Never Heard About VITAMINS” (*Parents’ Magazine* 1941; Apple 1996). The advertisement from which this sentence was abstracted was for a One-A-Day vitamin A and D tablet that prevented “poor teeth and bones.”

The era between the two world wars is where grandmothers, the traditional keepers of nutrition wisdom for infant growth in primitive cultures, became painted as nutritional nitwits and white-coated scientists or look-alike actors morphed into dietary gurus. Doctors, dentists, scientists, governments, professional organizations, and the food industry took charge of the nutrition-and-health message. Motherhood became equated with keeping up with the latest and the greatest vitamin knowledge that appeared in the popular press (Apple 1996). A mixture of vitamin science leading to Nobel Prizes, miraculous vitamin cures for deadly deficiency syndromes, and the relentless advertising for vitamin supplementation impacted sales and nutritional beliefs. Retail sales of vitamins in the United States grew from less than \$700,000 in 1925 to \$82.7 million in 1939 (Apple 1996).

Vitamin D played a role in creating the hypothesis that a healthy diet can be defined in terms of simple sums of required amounts of micro- and macronutrients. This nutritional hypothesis would be referred to in 1997 as *nutritionism* (Scrinis 1997). This magic of eating the daily requirement of essential nutrients regardless of one’s diet would become advertised first in the 1920s. The earliest standardized vitamin concentrate, marketed in Britain, was Ostelin, a vitamin D preparation of Glaxo Laboratories (today GlaxoSmithKline, London, UK) brought on the market in 1924 (Davenport-Hines & Slinn 1992). The first vitamin D–enriched breakfast cereal was a cereal licensed by the Quaker Oats Company

in 1927. Put your trust in biochemists rather than Mother Nature. Drink milk with 400 United States Pharmacopoeia (USP) units of vitamin D. This would slowly evolve into today's nutritional world, where processed food wrappers contain breakdowns on micro- and macronutrient content and organizations such as the Institute of Medicine determine dietary reference intake for vitamin D without considering cereal or sugar intake or assuming that Americans rarely are exposed to sun (Ross *et al.* 2010).

Vitamin D provided a powerful sales argument for the nutritionism hypothesis that even today finds traction.

The 1920s sales pitch for vitamin D was that living in the northern hemisphere is associated with many months of UVR darkness (Figures 36.2 and 36.3). If one believes in nutritionism (i.e., needing daily doses of essential nutrients for health) and mistrusts the process of natural selection, it may seem plausible, especially to supplement sellers, that a daily vitamin D dose is needed. This plausibility was exploited with skilful marketing (Figure 36.3). An infant formula advertisement by GlaxoSmith-Kline provides a first glimpse of how the emerging vitamin science would make infant formula look equal, if not superior, to breastfeeding (Copywriter 1920). In

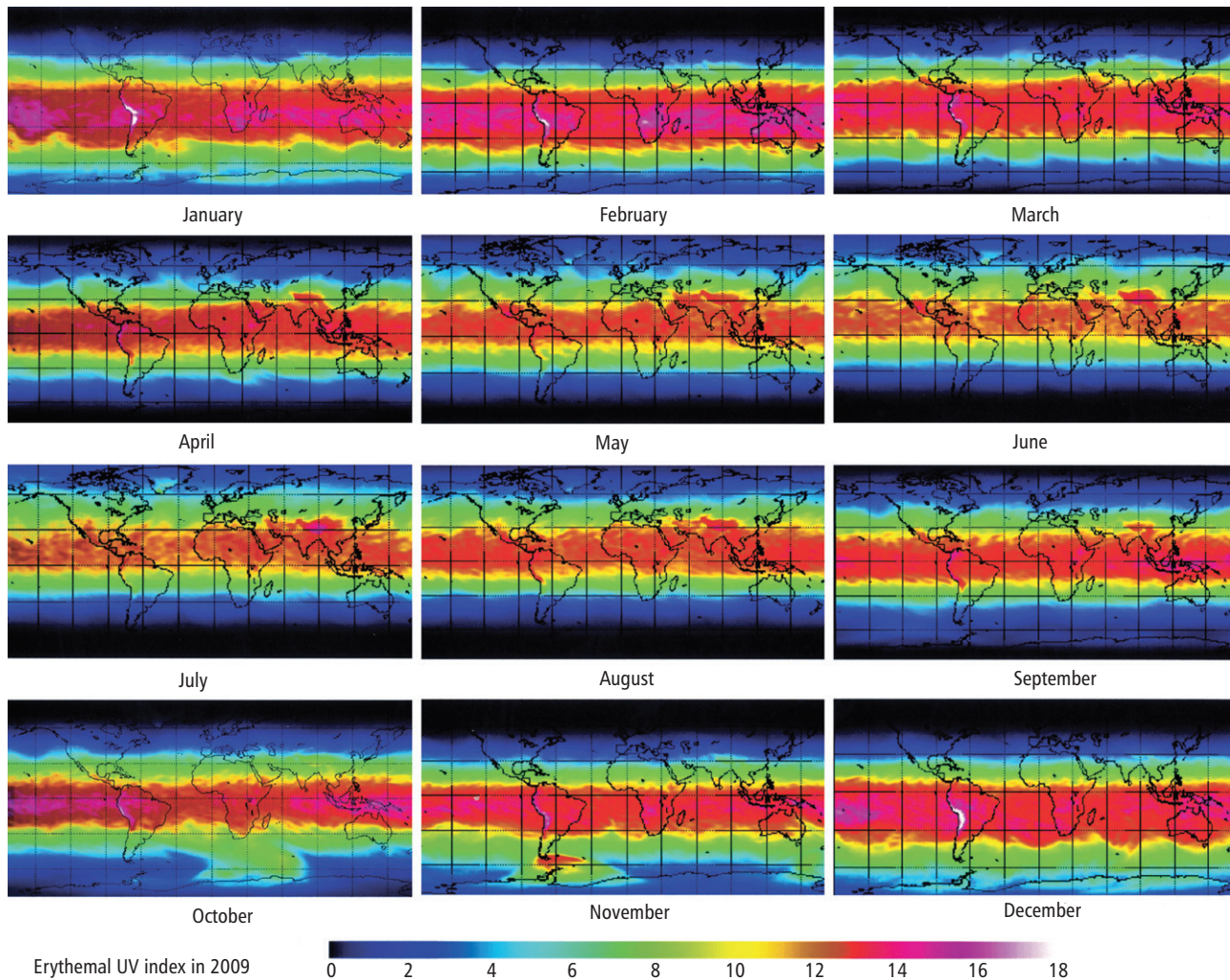


Figure 36.2 World map of monthly levels of ultraviolet rays (UVR) in 2009. The UVR levels are highest in the tropics (red and pink zones on map) and decrease in intensity with increasing latitude (deep blue and black zones on map). Most of Europe and North America have low UVR levels between November and March. The varying UVR levels are the primary driver of skin pigmentation, or lack thereof (Jablonski & Chaplin 2010). The endocrine vitamin D system was the selective survival force driving the creation of depigmented and tannable skin as hominids migrated out of the tropics (Jablonski & Chaplin 2010). Modern migration from ancestral high-UVR homelands into low-UVR countries causes a mismatch between the genetics of skin pigmentation and UVR levels. Such mismatches explain why over 80% of the US rickets cases in a recent survey were black (Weisberg *et al.* 2004). Traditional dietary habits and local food characteristics also correlate with UVR levels suggesting that the dissonance between genes and latitude through modern migration may go deeper than skin color. (Reproduced with permission from KNMI / TEMIS.)

Display Ad 301 -- No Title
 New York Times (1923-Current file); Mar 3, 1929;
 ProQuest Historical Newspapers The New York Times (1851 - 2006)
 pg. RP24



They need help *now* in building their bones and teeth!

"Give them Bottled Sunshine as regularly as milk and orange juice," baby specialists urge mothers.

Do you know that the kind of bones and teeth your baby builds during the first year or two of life has much to do with his health and appearance in later years?

Whether he will have a well-shaped head, well-formed jaws and chin, a deep, full chest and straight legs; whether he will have sound, even, uncrowded teeth that will not decay easily—these all depend on the kind of bones and teeth he builds now.

He needs your help, even though he is breast-fed. Even though he looks strong and healthy, he needs an unfailing supply of one essential factor—Vitamin D—in order to build strong bones and sound teeth from the food he eats.

This factor can be supplied from two sources. From sunshine and good cod-liver oil.

If you could be sure that your baby had enough sunshine on his bare skin, you could protect him in that way.

But under modern living conditions this is almost impossible. Clouds, fog, smoke, and clothing shut out the protecting ultra-violet rays of sunshine.

The simplest way to supply this needed factor is to give your baby good cod-liver oil regularly. Bottled Sunshine!

In pointing out this need to mothers, many physicians advise the use of Squibb's Cod-Liver Oil.

They recommend Squibb's because they know that the quality of the oil makes a difference in the results obtained. And they know that Squibb's Cod-Liver Oil is very rich in two vitamins.

It is very rich in Vitamin D which helps to build good bones and teeth, and in Vitamin A which promotes growth and increases resistance to many infections.

New! a mint flavor—so easy to take!

Here's something new, now, for expectant and nursing mothers, older children and grown-ups! **Mint-Flavored cod-liver oil!** It's a cool, fresh flavor so easy to take—it will appeal to even the most sensitive taste. You can get both kinds now at all reliable drug stores—Squibb's Plain and Squibb's Mint-Flavored Cod-Liver Oil.



It is almost impossible for your baby to get enough direct sunshine on his bare skin to protect him. Clothing shuts it out.



In Bottled Sunshine—good cod-liver oil—is the element of sunshine that helps to build good bones and teeth.

SQUIBB'S COD-LIVER OIL

PLAIN AND MINT-FLAVORED

*Produced, Tested and Guaranteed by E. R. Squibb & Sons, New York
 Manufacturing Chemists to the Medical Profession Since 1858*

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Figure 36.3 A 1929 commercial advertisement for vitamin D published in the *New York Times*. Vitamin D supplements promised to deliver over 10 health benefits including an increased resistance to dental decay. This claim was endorsed in 1946 by the American Medical Association (AMA) but not by the American Dental Association (ADA). The credibility of science (the element of sunshine that builds bones), the authority of medical professionals (the urging of baby specialists and physicians), the creation and exploitation of guilt feelings (your baby needs your help), and the instilling of fear (it is almost impossible to get enough sunshine) were combined into a powerful sales message: Buy it now! (Reproduced with permission from Bristol-Myers Squibb.)

just a couple of decades, such commercials turned infant formulas from a recognized scourge to the most common form of infant feeding (Thulier 2009). Nutritionism was born in the 1920s and has continued to thrive.

The 1920s: start of the unbridled marketing of vitamins

"It is certainly true that before commercial interests enter largely into this field, many more carefully controlled clinical studies should be made than are now available" (Eliot & Powers 1934). This quote originates from a 1934 *Journal of the American Medical Association* (JAMA) publication authored by two physicians expressing their concern over the emerging financial conflicts of interest related to food enrichment and fortification. Some claim that it was during the 1920s that commercialism began to drive science (Apple 1996) as opposed to science driving commerce. Vitamin D was patented, according to one person involved, to protect one food industry over another (Apple 1996).

The almost complete absence of checks and balances on commercial health claims during the Roaring Twenties may have played a part in creating a meme, a unit of cultural vitamin ideas, that would propagate and amplify in the public mind to what it is today. Vitamin D received the most publicity and had the most general sales appeal (Palmer & Alpher 1937). Commercial advertisements of this era suggested that well-shaped heads, well-developed jaws and chins, and sound teeth were connected to strong backs, straight bones, and full deep chests. Proper mineralization in infancy was promoted as predicting appearance and health throughout life (Figure 36.3).

It would be a mistake to think that carefully controlled studies supporting such commercial claims were never conducted before the Second World War. The causes of cholera and puerperal fever were determined by analytical epidemiologic studies in the 1850s. Similarly, intervention studies using pseudo-randomization, blinding, placebo controls, standardized examiners, and statistical analyses were conducted prior to the 1940s (e.g., Deverall and Reynolds 1936). The absence of such studies for vitamin claims was the choice of those making the commercial claims and *not* an inability to conduct such studies. Indeed, the clamoring of many scientists for the conduct of carefully controlled studies would remain vociferously opposed by some industry insiders, even today.

Few government regulations managed to restrict commercial vitamin health claims. The Federal Food, Drug and Cosmetic Act requesting evidence of safety was enacted in 1938 more than a decade after commercial

advertisements for vitamins began. Therefore, there was more than 10 years of freedom to mold and shape public opinion on the effects of mild vitamin deficiencies and the value of vitamin supplementation.

That is not to say that the Food and Drug Administration (FDA) or the Federal Trade Commission (FTC) did not attempt to regulate or rein in health claims. After the Second World War, several efforts were initiated. Proposals for regulation were issued, consensus was sought, and stakeholders were consulted, all to no avail. The US Congress twice enacted legislation that decreased—instead of increased—control over vitamin health claims. In 1976, legislation was passed declaring that natural and synthetic vitamins or minerals or their combination could not be classified as a drug and thus not regulated as such. One historian suggested that this was the first step backward in consumer protection since 1906 (Young 1992; Apple 1996). Subsequent efforts to regulate health claims led to an even bigger defeat with the 1996 Dietary Supplement Health and Education Act. A *New York Times* editorial referred to this legislation as the Snake Oil Protection Act (Editorial 1993). The FDA was left largely toothless to control commercial health claims related to supplements, even when hundreds of deaths and thousands of serious adverse events occurred (Hurley 2006). These small epidemics of deaths related to dietary supplements other than vitamins may have been chump change when compared with the death toll due to vitamin supplements.

Interpreting the few controlled studies conducted in support of commercial health claims in the early to mid-twentieth century is a treacherous exercise. Vitamin D, because of its scarcity in most foods and because of the patents protecting the fabrication of synthetic vitamin D, became co-opted by financial interests maybe more than any other vitamin (Apple 1996). Today we know that many design and analyses elements need to be in place to separate science from commercialism. Almost none of these were present for the studies on vitamin supplements prior to the 1980s. To what extent industry-funded studies suffered from selective publication, the absence of independent data and safety-monitoring boards, or the selection of contrived controls may never be known. Industry-funded studies are difficult to recognize, as disclosure of financial conflicts of interests was rare in those days. One investigator reported that low vitamin C intake, and not vitamin D, was the cause of dental caries, but his studies were supported by the California Fruit Growers Exchange (Hanke 1929; Palmer & Alpher 1937). Another investigator, a physician, reported that vitamin D decreased dental caries risk while eagerly seeking business sponsors and high-level consulting work from several food and pharmaceutical companies

that had vitamin D-fortified products in the pipeline (Davenport-Hines & Slinn 1992).

Few studies, sketchy regulation, and potentially compromised science competed with sophisticated marketing campaigns. The US budget for food advertisements in 1935 exceeded \$38 million (Palmer & Alpher 1937). The vitamin science would Teflon-protect companies against quackery allegations. Vitamin D advertisements showed doctors in white coats, X-rays of bones, and occasional endorsements from the American Medical Association (AMA). The word *science* was sometimes spelled with a capital “S” like a religion, and who, after all, could disagree with the deadly nutritional deficiency syndromes that just decades earlier had plagued selected populations (Figure 36.3)? The use of the “natural” claim may have been the most treacherous advertising claim. If vitamins are found in nature, they must be safe. The sunlight craze in the early twentieth century would make the “sun-in-a-bottle” argument attractive; what can be more sexy and safe than sunlight? The logic of this argument would evaporate when it became clear that vitamin D supplements can lead to death while overproduction of vitamin D through sunlight is physiologically impossible (Jablonski & Chaplin 2010).

Processed versus unprocessed foods: an early twentieth-century conflict

When you take tryptophan in pills or a bottle, it's not natural. Never in man's evolutionary history did he or she take an individual amino acid. The body does not handle it in the same way the body handles tryptophan in a protein. The body cannot use it, for instance, to make its own protein. So tryptophan, in spite of being called a nutritional supplement, has nothing whatsoever to do with nutrition. Tryptophan is a drug. (Hurley 2006)

Thus spoke Dr. Richard Wurtman, professor of basic neuroscience, Massachusetts Institute of Technology (MIT); physician at Harvard Medical School; and director of MIT's Clinical Research Center, when he testified at a congressional hearing on July 18, 1991. The logic of his argument has far-reaching implications as humans have never in evolutionary history consumed vitamin D, calcium, or sugar in a gummy bear, pill, or bottle.

Two competing hypotheses on nutrition and health emerged amid the unfettered health commercialism of the early twentieth century. The first hypothesis postulated that fortified and enriched processed foods are the rational choice for a modern world. Thanks to miraculous nutritional science, processed food could be enriched, fortified, or combined with dietary supplements. Humans would trade in breastfeeding for bottle

feeding, whole foods for processed foods. Some of the hypothesized health benefits of processed foods were laid out in a 1980 nutrition textbook (el Lozy *et al.* 1980) that was coauthored by the founding father of the nutrition department at Harvard University, Frederick Stare, who was described as the United States' leading nutritionist (Staff Writer 2002):

Thus it makes good sense . . . to increase the production and dietary use of the most efficient and least expensive source of calories . . . the chemist can improve many of our basic foods by means of appropriate fortification . . . with fortification, food habits do not have to be changed, a very difficult and time-consuming task . . . we must consume fewer animal foods and more cereals, potatoes, and sugar, the latter being only calories, but yet the most efficient and least expensive source of calories. The proper use of pesticides, antibiotics in animal feed, and food additives to prevent spoilage of foods is vital to producing enough food.

The gradual disappearance of nutrition courses in medical and dental schools is a logical consequence of the hypothesis that “food habits do not have to be changed.” Adverse effects of lifestyle choices such as cigarette smoking could be neutralized through fortification, supplements, and drugs. “Prescription, not proscription,” became the hypothesis of the late twentieth century. Rachitic infants need vitamin D supplements, not dietary changes or advice to play in the sun. In the 1990s, it became clear that this “Prescription, not proscription” failed when it came to preventing the harmful effects of cigarette smoking. Vitamin A supplements, the much-touted antioxidant wonder drug, increased rather than decreased lung cancer mortality (Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group 1994).

The alternative hypothesis postulated that the chronic diseases of civilization are the result of a mismatch—a dissonance—between nutrition, geography, and our genetic makeup (Cleave & Campbell 1996; Eaton 2007; Jablonski & Chaplin 2010). According to this hypothesis, our genetic and epigenetic machinery is incompatible with vegetable oils and trans fats, refined carbohydrates, and dietary supplements. Similarly, modern migrations to lower UVR regions caused a dissonance between skin pigmentation and UVR levels that lead to a handicapped endocrine vitamin D system. Weston Price, an early skeptic of nutritionism, described our lack of understanding with respect to synthetic vitamin D:

There is marked tendency in modern civilization to substitute synthetic products for Nature's foods. This has been particularly true of vitamins. While these tendencies are fortunate so far as Nature's requirements can be

duplicated, the limited knowledge regarding the number and kind of activators and their individual roles in conjunction with other necessary activating substances and body building mineral requirements has resulted in serious injuries and some misapprehensions. (Price 1942)

Public health interventionists waded into these debates and influenced how foods ended up on our table. Weak evidence, the quintessential characteristic of most nutritional advice, would guide nutritional mandates for populations. Thiamine became part of the enrichment of US flour, even though frank thiamine deficiency and the consequent beriberi were endemic in Asia, not in the United States. Most recently, cereal products mandated to be fortified with synthetic folic acid supplement to provide a potential benefit for pregnant women, who constitute about 1% of the population, create concerns related to cancer (Ulrich & Potter 2006), asthma, and allergy (Ownby 2009) for the remaining 99% of the population.

Disagreements on such nutritional policies or recommendations were common almost from the beginning of “vitamania” (Apple 1996). McCollum, the foremost nutritionist in the United States and discoverer of vitamin A, B, and D, was fired from the panel deciding on flour enrichment and fortification because he was “strongly critical” (Simoni *et al.* 2002). Disagreements on mineralizing factors were equally contentious. Different government agencies within and across countries reached opposite conclusions (Apple 1996). For instance, fortified breakfast cereals were considered healthy in one country but sufficiently damaging to children's livers and kidneys in another country such that they were banned (Meikle & Harding 2004). The spokesperson for the cereal company reported that they were “Gobsmacked that the benefits we have been putting in our cereals for 70 years have been called into question” (Phagura 2004). And, indeed, the spokesperson was correct: boardroom nutritional wisdom was rarely questioned, let alone submitted to randomized trials, during the twentieth century.

Late twentieth-century defeat of the unprocessed food hypothesis and the advent of osteoporosis

Yudkin was ridiculed for his advocacy of the hypothesis that sugar causes heart disease. . . . And anybody else who said something bad about sucrose, they'd say, “He's just like Yudkin.” (Taubes 2007)

This excerpt from Gary Taubes's book *Good Calories, Bad Calories* (2007) details the weak science that led to

the twentieth-century rise of the high-carbohydrate and, according to Mellanby's experimental work, rachitic diet. In 2009, more than 50 years after Yudkin published his research, the American Heart Association recommended to limit sugar intake (Johnson *et al.* 2009).

The unprocessed food hypothesis became marginalized in the second half of the twentieth century. Physicians, nutritionists, and researchers who espoused evolutionary hypotheses were steamrolled over, forgotten and ignored, or painted as quacks (Yellowlees 1991; Campbell 1996). Modern industrial foods, which appeared in the “evolutionary wink of an eye” (Cleave & Campbell 1996), were declared the foundation of the healthy-food pyramid. The foremost nutritionist in the United States described a sugary drink as “a healthy between-meals snack” (Staff Writer 2002). Sugar, the prototype example of an ultra-processed food, was considered a heart-healthy snack.

Public health advice on dietary fats and sun and a steadily increasing intake of dietary phosphates may have had a nasty side effect on bone health. It seemed as though public health officials did everything possible, based on the best scientific evidence, to sabotage natural vitamin D metabolism. Butter, cheese, and eggs rich in Vitamin D, A, and K; minerals; and other micronutrients became regarded with suspicion and traded in for vegetable oils. High-carbohydrate cereals would replace the vitamin- and mineral-rich organ meats. This was a first blow to bone health as experimental animal research indicated that such nutritional advice led to bone demineralization.

The second blow to bone health was that sunlight, the natural vitamin D generator, was put in the same carcinogen class as cigarettes. The sun became demonized: “A healthy tan: you may as well smoke a cigarette” (Mott 2010). The public health advice was to trade in a natural tan for spray tans, apply sunscreens, or avoid the sun during those times of the day that vitamin D is actually generated. Victoria Beckham's spray tans became described as a role model for sun-smart behavior (Drosu 2009).

The third blow for bone and dental mineralization was the steadily increasing intake of dietary phosphates such as those present in many carbonated, sugary drinks. Increased dietary phosphates may depress vitamin D levels through the FGF-23 pathway.

The increasing popularity of sun safety, low fat, and high dietary phosphate intake coincided with the arrival of osteoporosis. Osteoporosis became the Johnny-come-lately disease of civilization. It was recognized in 1984 as a serious public health threat in the United States (Peck *et al.* 1984). Several osteoporosis associations, such as the National Osteoporosis Foundation and the National

Osteoporosis Society, were founded in the 1980s. In the United States toward the end of the twentieth century, serum vitamin D levels dropped (Looker *et al.* 2008) and forearm fracture rates in the young increased (Khosla *et al.* 2003). The number of hip fractures increased five-fold between 1920 and 1996 (Melton *et al.* 1998), and rickets made a comeback appearance in the twenty-first century (Abrams 2002). This late arrival of an osteoporosis epidemic may not be surprising considering that osteoporosis today is regarded as a pediatric disease—a disease best prevented by proper mineralization in early life. Osteoporosis may well be the disease with the longest latency period among the diseases of civilization.

Regardless of whether there is a cause-and-effect relationship between public health advice and the appearance of an osteoporosis epidemic, the hopes for bone



Figure 36.4 A reproduction from a 1980 nutritional textbook showing twins with rickets (el Lozy *et al.* 1980). The caption associated with this picture informed the reader that these markedly obese twins were first-prize winners at a baby show and had been well nourished on artificial feeding (el Lozy *et al.* 1980). It is striking that the medical authors of this textbook equated obesity with well nourishment and that baby-show judges equated fat with beauty. Pediatric obesity appeared to be a source of pride rather than an early warning for the emerging diabetes and obesity epidemic. (Reproduced with permission from Pfizer, Inc.)

health increasingly relied on drugs and dietary supplements. The dietary supplementation industry, the medical and pharmaceutical industry, the sun care industry, and the sun spray manufacturers could not have written a better script to create market share. Sunscreen sales soared (Staff Writer 1993), vitamin D testing skyrocketed (Staff Writer 2008), and one out of five women took prescription bone medication (Dawson-Hughes *et al.* 2002). More than 40% of the adult US population now takes calcium supplements, vitamin D supplements, or both (Bailey *et al.* 2010). Whether or not this approach of combining a vitamin D—robbing lifestyle with mineralizing supplements leads to overall reduced morbidity and mortality remains largely untested.

Randomized controlled trials of dietary supplements: is it too late to change beliefs?

Before one interferes with the peace of mind and habits of others, it seems to me that the scientific evidence—the exact weight of the evidence free from emotion—should be rather carefully examined. (Fisher 1958)

Sir Ronald Fisher (1890–1962), geneticist, statistician, and evolutionary biologist, has been described as “a genius who almost single-handedly created the foundations for modern statistical science” (Hald 1998).

Most scientists, regardless of their hypotheses, agree that pivotal randomized trials on mortality and morbid-

ity are the gold standard for evaluating the impact of dietary supplements. Disease-specific endpoints such as bone fractures—or, worse, surrogates such as bone density—can be misleading.

Randomized controlled trials in the twentieth century demonstrated that vitamin and mineral supplements do significantly impact mortality or morbidity, but probably not in the way that was anticipated (Table 36.1). A systematic review of trials suggests that supplements with beta-carotene, vitamin A, vitamin E, or vitamin B₆ and B₁₂ significantly increase mortality risk (Bjelakovic *et al.* 2007). Based on the best available evidence, the possibility cannot be excluded that vitamin C supplements similarly raise the mortality risk. Calcium supplements without co-administered vitamin D supplements significantly increase the risk for cardiovascular disease (Bolland *et al.* 2010). There is a nonsignificant trend indicating that calcium supplements increase mortality (Bolland *et al.* 2010).

The implications of these randomized controlled trial results are ominous. Calcium supplement use by 50% of the 35- to 74-year-old US population would cause 53,000 myocardial events annually. Calcium supplement use by 50% of US women 65 years and older would cause 39,000 hip fractures annually. Vitamin A supplement use by 30% of the US population would cause 110,000 deaths annually. In 2007 in the United States, there were 301,621,157 people and 803.6 deaths per 100,000 indi-

Table 36.1 Mortality risk and fracture risk associated with vitamins and calcium. The results presented are those from the most recent systematic reviews. All vitamins, when taken as a supplement, have been associated with an increased mortality risk. A combination of vitamin D and calcium may be associated with a small, marginally significant, reduced fracture risk. Three out of five studies so far have identified increased mortality risk associated with high serum levels of vitamin D, which suggests that randomized trial evidence regarding the effect of vitamin D on mortality is of the utmost importance to public health recommendations (Bischoff-Ferrari *et al.* 2007; Bjelakovic *et al.* 2007; Boonen *et al.* 2007; Reid *et al.* 2008; Avenell *et al.* 2009; Ebbing *et al.* 2009; Mahomed & Gülmezoglu 2009; Abrahamsen *et al.* 2010; Bolland *et al.* 2010; Winzenberg *et al.* 2010).

Supplements	Overall mortality risk		Bone fractures
	Number of randomized controlled trials	Mortality risk ^a	Adults and seniors
Beta-carotene	12	+7% (+2% to 11%)	
Vitamin A	5	+16% (10% to 24%)	
Vitamin E	26	+4% (1% to 7%)	
Vitamin C	13	+6% (–6% to +20%)	
Vitamin B6 and B12	2	+18% (4% to 33%)	
Vitamin D	3	Increase?	Hip fracture: +10% Any fracture: +1%
Calcium	7	+9% (–4% to 28%)	Hip fracture: +57%
Vitamin D and calcium	4	Increase?	Any fracture: –8% (–1% to +14%)

^aRisk and 95% confidence intervals.

viduals. If the relative risk associated with vitamin A is 1.16 (95% confidence interval: 1.10–1.24) and 30% of the population is exposed, the population attributable risk is ~5% and the population impact number over 1 year is ~111,000 (data from <http://www.cdc.gov/nchs/facstats/deaths.htm> and <http://www.census.gov/popest/states/NST-ann-est2007.html>). Despite such statistics, the “intense marketing with a contrary statement” (Bjelakovic *et al.* 2007) continues, and public health missionaries keep advocating nutritional policies on supplements without pivotal randomized controlled trial evidence. Are the vitamin memes created in the 1920s so virulent that no amount of randomized controlled trial evidence will remove the cognitive dissonance?

How do supplements affect bone health outcome measures? This question is secondary because it may provide a misleading answer to the more important question of reduced morbidity and mortality. This secondary question appears somewhat inconsistent with commercial claims (Table 36.1). Two systematic reviews concluded that calcium supplements make hip bones 50% more susceptible to fractures (Bischoff-Ferrari *et al.* 2007; Reid *et al.* 2008). Three systematic reviews came to the conclusion that vitamin D supplements in the form of injections or supplements increased fracture risk by a nonsignificant 1% (Boonen *et al.* 2007; Avenell *et al.* 2009; Abrahamsen *et al.* 2010). The most recent randomized controlled trial showed a significantly increased fracture risk and a significantly increased fall risk (Sanders *et al.* 2010).

Currently, the evidence in favor of supplementation comes from trials in which vitamin D and calcium supplements are taken simultaneously. This supplement combination is associated with an 8% reduced fracture rate with a statistically significant *p* value of 0.025. Considering that no adjustments for multiple hypothesis testing were made, this *p* value may be considered marginal and in need of independent confirmation (Abrahamsen *et al.* 2010). The key question will be whether vitamin D supplements or the combination of vitamin D and calcium supplements increases mortality risk as almost every other vitamin supplement does. The answer to this question is currently being investigated by the *Cochrane Collaboration*. The emerging findings are conflicting. There currently are three studies that report an increased mortality risk at higher serum vitamin D levels (Ross *et al.* 2010).

Dental diseases: caused by darkness and malnutrition?

You all know the story of the cod and the halibut—how they have laid down their lives in order to preserve the teeth of western man. (Brekhus & Armstrong 1936)

This comment reflects early signs of skepticism in the dental community of the claims that vitamin D is important to dental health. After the Second World War, fluoride began its slow rise in popularity and the evidence on vitamin D was tossed in the dustbin of history.

Research in the early twentieth century linked rickets to a variety of dental conditions including dental caries, malocclusions, hypoplastic teeth, caries-like lesions, horizontal periodontal bone loss, fracture of teeth, and pyorrhea (McCollum *et al.* 1922b; Mellanby 1926, 1940). One striking aspect of these dental-systemic associations was the link between rickets and dental malocclusions. Weston Price, the founding member and head of the scientific council at the American Dental Association (ADA) from 1914 to 1928, observed that a switch from traditional to industrial foods can lead to narrowed dental arches within one generation (Price 1940). The plausibility of his observations appears to be supported by current studies that relate vitamin D-resistant rickets, a genetic condition, to malocclusions (Pereira *et al.* 2004; Al-Jundi *et al.* 2010; Figure 36.1).

The hypothesis that dental diseases were nutritional deficiency syndromes just like rickets became incompatible with clinical observations. Rickets largely disappeared from Western civilization, but caries remained rampant. Caries was highly prevalent in individuals living in sunny countries. Whatever cured rickets did not seem to affect dental caries rates. Similar arguments were developed to dismiss the hypothesis that periodontal diseases are a nutritional deficiency syndrome caused by vitamin D deficiency. The hypothesis on etiology changed from caries as a deficiency disease to the hypothesis of caries as a chronic disease with a complex etiology that includes vitamin D as one component cause. The evidence in support of this hypothesis has been growing. Association studies related osteoporosis and vitamin D nuclear receptor alleles to destructive periodontal disease and tooth loss (Hennig *et al.* 1999; Tachi *et al.* 2001; Inagaki *et al.* 2003; de Brito Junior *et al.* 2004; Geurs 2007), sunshine to dental caries (Mills 1937; East 1939; Hadjimarkos *et al.* 1950; Powell 1983), and vitamin D intake or serum levels to dental health (Krall *et al.* 2001; Dietrich *et al.* 2006; Dixon *et al.* 2009; Miley *et al.* 2009). The largest body of experimental evidence on vitamin D relates to dental caries and predates World War II. Approximately a dozen studies in five countries focused on vitamin D₂, vitamin D₃, and natural photo isomers of vitamin D₃ induced by UVB light. Four mechanisms were hypothesized by which vitamin D lowered caries risk: (1) better tooth development and thus more resistance to caries, (2) a more vigorous dentinal response leading to caries arrest, (3) a topical fluoride-like effect (McBeath & Verlin 1942), and (4) an improved

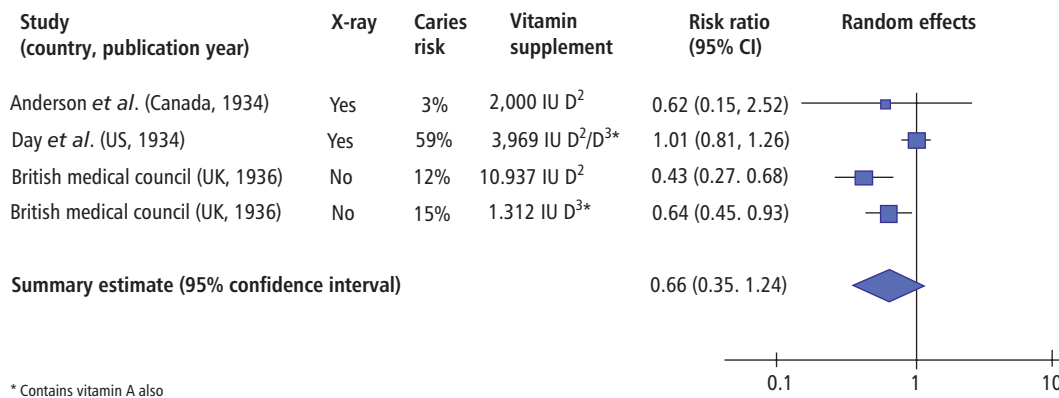


Figure 36.5 Systematic review on the effects of vitamin D supplementation on caries risk in erupting teeth. Vitamin D2 and D3 are respectively synthetic and natural vitamin D. On average, vitamin D decreases caries risk nonsignificantly by 34% (RR = 0.66; 95% confidence interval: 0.35–1.24). This overall estimate needs to be interpreted with caution due to the significant heterogeneity between studies. Note the substantial variability in caries risk, vitamin D dosage, the presence of other active ingredients in the supplements such as vitamin A, and caries examination methodology. Confidence intervals were inflated by 29% to correct for within-patient clustering of caries risk.

immunological response mediated through saliva (McCullum 1939). More recently, it was hypothesized that UV radiation could affect dental decay through improved salivation independent of vitamin D and immune function (Hargreaves & Thompson 1989).

The majority of these caries studies reported positive results. We focus here on the four studies (Anderson *et al.* 1934; Day & Sedwick 1934; Medical Research Council of Great Britain 1936) that reported separately the effect of vitamin D on erupting teeth (Figure 36.5). A statistical summary of these studies suggests a non-significant 34% decrease of caries risk associated with vitamin D.

These promising findings of nutritional vitamin D supplementation to combat caries became largely forgotten after World War II. A few exceptions appeared in the literature such as (1) an ecological correlation between multiple sclerosis and dental caries that suggested a vitamin D deficiency as a common causal link (Craelius 1978), or (2) a reduction of caries associated with UV radiation (Ott 1975; Hargreaves & Thompson 1989). Nonetheless, current dental textbooks no longer refer to vitamin D as a caries preventive agent (Newbrun 1989) or a craniofacial growth modifier, or report explicitly that there is no relationship between vitamin D deficiency and periodontal disease (Newman *et al.* 2006).

The current renewed interest in vitamin D and general health may lead to a renewed interest in the effects of vitamin D on dental health. For instance, there is an NIH-funded clinical trial underway to evaluate the effect of vitamin D supplements on experimental gingivitis (Boston University 2010). The larger hypothesis of how

mineralizing factors in nutrition affect dental health currently remains a largely unexplored topic in dental research.

Conclusions: evolutionary health promotion?

A proper respect for scientific method requires that when enormous sums of money, hundreds (if not thousands) of painstaking investigations and untold hours of human efforts have produced contradictory, inconclusive results (as disease incidence increases), it is appropriate to at least consider a different approach. In Kuhnian terms, it may be time for a paradigm shift. (Eaton 2007)

The nineteenth- and twentieth-century history was characterized by mass nutritional experiments conducted without informed consent or randomized controls and often driven by commercial rather than scientific interests. An increase in the consumption of processed foods caused a first wave of endemic nutritional deficiency syndromes. This disaster was solved through supplementation with vitamins in the early twentieth century. This may have been a pyrrhic victory as processed enriched foods may have caused a second wave of chronic diseases of civilization. It is true that billions of dollars in research investment led to the identification of some partially successful treatments and antidotes to poor nutrition such as statins, antacids, fluorides, or antidiabetes medications, but the bill is exorbitant. In 2006 alone, the expenditures for dental disease (\$90 billion) exceeded the cost of the five most costly diseases (Soni 2009).

One would think that the emergence of evidence-based medicine would make public health officials more cautious evangelizers. However, messianic messages, still unsupported by good evidence, keep appearing. The current hysteria about sunlight exposure may be the latest example of public health policy driven by questionable evidence. Skin pigmentation has been described as the perfect model of evolution and natural selection (Jablonski & Chaplin 2010; Figure 36.2). Yet parents are advised that their infants and children should lead a vampire-like existence, effectively disabling the sun-dependent and exquisitely sensitive endocrine system that has evolved over hundreds of thousands of years. Tanning, which provides an evolutionary survival advantage for depigmented skins, is regarded as a marker of unhealthy behavior. Not even sun avoidance for the myopic purpose of melanoma prevention appears to be consistent with the evidence. A systematic review suggests that chronic sun exposure nonsignificantly reduces melanoma risk (Gandini *et al.* 2005) and that it may lower the risk of other cancers more effectively than vitamin D supplements (van der Rhee *et al.* 2006; Rhee *et al.* 2009). Sun in early life has been related to other beneficial effects such as a reduction in multiple sclerosis risk (Hayes & Donald Acheson 2008). “Scaring the living daylight out of people” may indeed be “highway robbery” (Gillie 2010) and a return to the “dark ages” (Hassed 2002).

A paradigm shift is in order: a shift from antidotes to primary prevention, from specific diseases to overall health, from arguments that ignore genetics to evolutionary arguments, and from lesser evidence to evidence acquired through pivotal randomized trials. There should be a moratorium on public health advice that is not supported by pivotal clinical trials and that conflicts with evolutionary principles. Evolution, biology, biochemistry, clinical trials, and pivotal studies of the twentieth century support the hypothesis that the latitude-appropriate diet and skin color (Cordain *et al.* 2005) that promote the mineralization of bones and teeth also prevent the diseases of civilization. The need to test the evolutionary hypotheses that may have been first formulated half a century ago is overdue (Cleave & Campbell 1996).

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Clinical correlate: vitamin D deficiency

Ana Lucia Seminario and Elizabeth Velan

Vitamin D represents a group of fat-soluble secosteroids (molecules chemically similar to steroids except for one “open” ring), with D₂ (ergocalciferol) and D₃ (cholecalciferol) being the most relevant vitamins of the group. While vitamin D₂ is synthesized by plants (fungus/yeast), Vitamin D₃ is produced by humans in the skin when it is exposed to ultraviolet-B rays from adequate sunlight or artificial sources (Adams & Hewison 2010).

Once vitamin D is present in the bloodstream, it is first activated in the liver and then in either the kidneys or the immune system via monocytes and macrophages. If the active form of vitamin D (hormone calcitriol) is synthesized in the kidneys, it regulates the concentration of phosphate and calcium in the bloodstream by enhancing its absorption in the intestines. If calcitriol is synthesized by monocytes and macrophages, this hormone behaves as a cytokine protecting against microbial infections (Holick 1999).

Physiologically, the importance of vitamin D relies on its ability to maintain intra- and extracellular calcium and phosphate concentrations in order to preserve essential metabolic functions such as the promotion of healthy mineralization, growth, maintenance, and remodeling of the bone (Institute of Medicine 2010). Research has demonstrated the relationship among vitamin D and several acquired or inherited conditions. A representative selection of these illnesses includes, but is not limited to, rickets and osteomalacia.

Rickets is a childhood disease caused by a vitamin D–deficient diet, a lack of sunlight, or both, or genetic disorders, and it is characterized by delayed growth and/or deformity of long bones. Due to successful efforts in manufacturing vitamin D–fortified milk in industrial-

ized countries, this condition is mainly observed in developing countries or in latitudes with cyclic, seasonal lack of adequate ultraviolet-B rays from sunlight (Zargar *et al.* 2000). Based on the type of cause of the deficiency, treatment includes active forms of vitamin D supplementation as well as calcitriol.

Osteomalacia is an adult disease characterized by a general softening of bone causing proximal muscle weakness and bone fragility (Parfitt 1990). It is found in individuals with decreased absorption of vitamin D or inadequate sun exposure; patients with gastric or intestinal absorption conditions or surgeries, aluminum-induced bone disease, chronic liver disease, or kidney disease; and elderly patients with vitamin D–deficient diets. Treatment for osteomalacia is based on the primary cause of the disease and consists of supplementation of vitamin D and phosphate-binding agents, orthopedic surgical interventions, and pain control.

Other conditions related to vitamin D deficiency include, but are not limited to, familial hypophosphatemia, a rare inherited disorder with an impairment of phosphate transport in the blood and diminished vitamin D metabolism in the kidneys; hyperparathyroidism; hypocalcemia due to hypoparathyroidism; and osteoporosis, among others (Drezner 2007).

The impact of vitamin D deficiency in the mouth includes:

- Dental deformities
- Delayed formation and eruption of teeth
- Abnormal radiolucency and loss of cortical bone of jaws (in radiographs)
- Decreased muscle tone

- Defects in the structure of teeth (especially hypoplastic lesions in the enamel)
- Large pulp chambers
- Abnormal dentine calcification prone to pulpitis with multiple and apparently spontaneous dental abscesses
- Decreased buffer effect of the saliva (due to lack of an appropriate calcium absorption)
- Increased risk for dental caries (Souza *et al.* 2010)

Dental management is based on individual risk assessment and includes but is not limited to pain and anxiety control with local anesthesia, conscious sedation, and general anesthesia; preventive dentistry, since even minimal caries or attrition can lead to pulpitis; comprehensive preventive care; and prophylactic occlusal coverage. Due to the reduced mineral content of the bone, osteointegration could be compromised; therefore, dental implants are not recommended (Chaussain-Miller *et al.* 2003).

The case report presented in this chapter describes the consequences of vitamin D deficiency in the normal development of a child, its impact on the oral tissues, and the interdisciplinary effort needed to ensure successful medical and dental outcomes.

Case presentation

HN, an eight-year-old Caucasian male, presented to the Dentistry Department at Seattle Children's Hospital for a new patient exam accompanied by his mother. The mother's chief complaint was HN's severe dental crowding. She wanted to investigate the possibility of her son receiving fixed orthodontic treatment.

HN was born at 41 weeks via C-section due to intrauterine stress and was diagnosed with mild hypothyroidism, hypotonia, polycythemia, and thrombocytopenia. Soon after his birth, HN developed a lactose allergy, and by 15 months of age, he was diagnosed with developmental delays with distinctive facial features. Due to his medical background, HN has been constantly monitored and treated for several conditions that emerged as he grew. These undesirable outcomes included skeletal dysplasia, bilateral nasal duct obstruction, otitis media, mild hearing loss, speech delay, bilateral cryptorchidism, pervasive developmental disorder, vitamin D deficiency, and Ohdo syndrome. By the time of HN's first dental examination, he was taking levothyroxine, clonidine, and prevacid daily.

During his first dental evaluation, HN was very uncooperative (Frankl scale -/-); however, with his mother's assistance, it was possible to proceed without physical restraint. A brief examination was performed extraorally. He presented with a straight facial profile with no scars, temporomandibular joints that were within normal

limits, and general hypotonia of masticatory muscles with open bite and mouth breathing present in patients with vitamin D deficiency. Intraoral examination revealed a prominent tongue, although the floor of mouth, palate, and buccal mucosa were within normal limits. There was generalized gingival inflammation and bleeding during tooth brushing. HN had crestal attachment of the frenum at teeth 8 and 9, and his oral hygiene was poor. His occlusion was in the mixed dentition stage with an anterior crossbite, a Class III molar relationship with severely crowded arches both anteriorly and posteriorly. No caries was noted during this evaluation.

Craniofacial experts and orthodontists specializing in care for children with special healthcare needs were included in the patient assessment. After HN's occlusion needs were evaluated, a treatment plan that combined dental and orthodontic care was developed. It included a comprehensive dental assessment (clinically and radiographically), preventive and restorative dentistry and occlusion management under general anesthesia. During the session, small carious lesions were found in the permanent molars (restored with amalgam) and all four primary canines were extracted due to a severe lack of space. Dental calcification associated with vitamin D deficiency was observed radiographically in the chambers of the permanent molars (Figure 37.1A–B). Sealants were placed where appropriate. After two weeks of follow-up, the healing process appeared to be satisfactory, oral hygiene at home improved dramatically, and HN's parents were very satisfied with the results.

In the following years, HN returned to the clinic for recall examinations and evaluation of his occlusal needs. His behavior improved over time, but passive physical immobilization was used for his, and the dental team's, protection. Six years after his first dental evaluation, HN was placed again under general anesthesia for extraction of a deformed bone-impacted permanent canine (Figure 37.2C), a morphologic anomaly associated with vitamin D deficiency, and comprehensive dental care was performed (Figure 37.2A–B). Sealants were placed on all of his second permanent molars, his right permanent canine was extracted, and a full curettage of the follicle was performed. Even though third molars could be assessed radiographically as impacted, the oral surgery team recommended monitoring these teeth to determine the optimum time for extraction. Due to HN's behavior, no fixed orthodontic treatment was recommended.

At age 16, HN was a sweet young man with no new medical problems; however, he had a long list of medications that included daily doses of Levothyroxine, Omeprazole, Abilify, Vitamin D3, Risperidone, Benzotropine, and MiraLax. Allergies to amoxicillin and penicillin

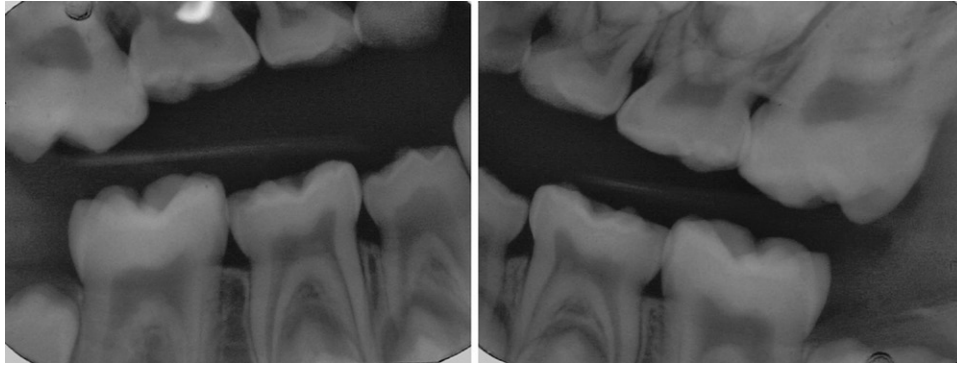


Figure 37.1 Radiographic presentations of patient HN at eight years of age with vitamin D deficiency.

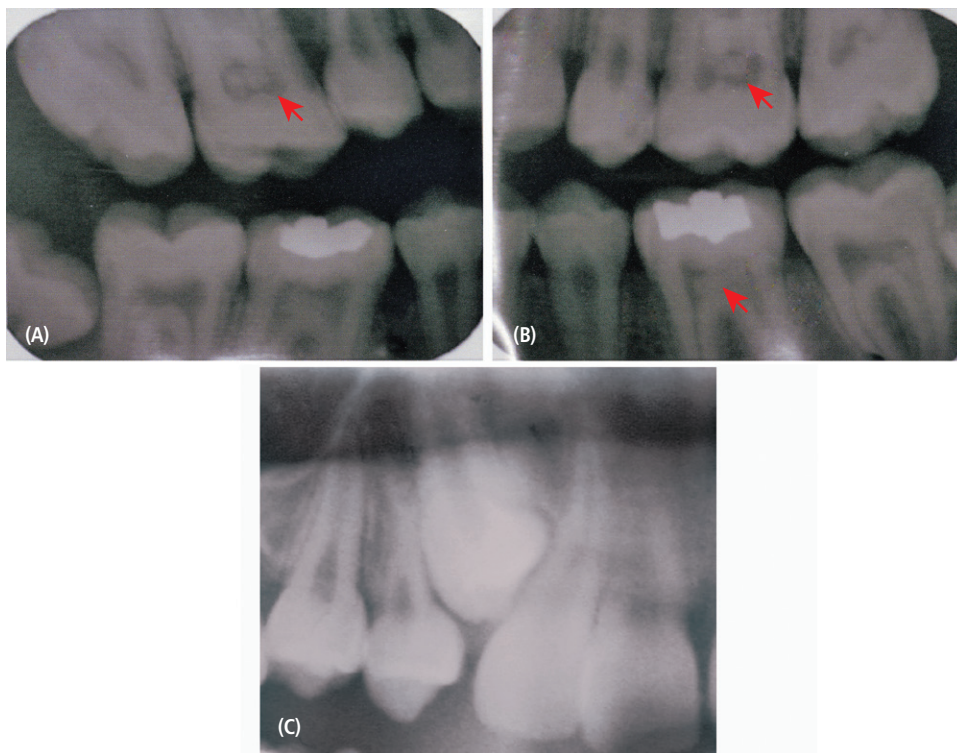


Figure 37.2 Radiographic presentations of patient HN at 14 years of age. A–B: Note the presence of calcifications in the pulp chambers of the molar teeth. C. Radiograph showing impacted and morphologically deformed tooth 6.

were added to his list of allergies. During HN's latest dental appointment, physical immobilization was not required, although it was difficult at times for him to hold still during certain aspects of the examination. The success of the appointment was made possible by constant positive reinforcement. As HN has grown, it has become more challenging for his mother to manage his oral habits; however, his oral hygiene was average with mild to moderate gingivitis present in localized areas (Figures 37.3). Some incipient carious lesions were

observed but without cavitation. When HN is re-evaluated and the oral surgeon recommends extracting HN's third molars under general anesthesia, a new comprehensive exam and treatment will be performed jointly.

Discussion

Vitamin D deficiency is not a disease in itself. It is a condition that leads to a series of undesirable health outcomes affecting normal development. The earlier it



Figure 37.3 A. Photograph of the maxillary arch of patient HN at 14 years of age. B. Photograph of mandibular arch of patient HN at 16 years of age. Note evidence of mild plaque induced gingivitis in both photographs.

is detected, the better the prognosis as long as the cause of the deficiency is properly determined.

The challenge of maintaining adequate oral health in children with special healthcare needs requires an interdisciplinary medical and dental team that is able to address care for these patient. In order to ensure acceptable treatment outcomes, the team must understand how each disease impacts oral tissues and then be able to determine how treatment may cause transitory or permanent changes in the mouth, especially in the developing dentition.

This case presents the typical scenario of a child with special healthcare needs for whom the ultimate goal was to maintain satisfactory oral health while he was growing up. The patient is starting his adult life with minimum restorative treatment to his permanent dentition and a good prognosis for maintaining this. One of the key factors in his success was the presence of consistent dental care via a “dental home.” The fact that his mother immediately understood the importance of oral health and put the tools in place to provide the necessary care had obvious positive consequences. Success was also due

to the excellent communication skills of the dental team and the patient’s family. Patients with a high risk of developing dental caries need frequent monitoring, and parents need constant positive reminders of how to manage their children’s oral health as well as prepare them for expected changes during the natural growth and developmental process.

Vitamin D deficiency has several oral manifestations. Its severity depends on the time at which the deficiency occurs. The earlier it occurs, the greater and deeper are the undesirable oral consequences. Although the literature regarding vitamin D deficiency and its oral consequences is limited, all healthcare providers agree on the importance of having a consistent “dental home” and the success of preventive dentistry (Goodman *et al.* 1998).

Conclusion

Vitamin D deficiency at a young age may have severe consequences on the normal growth and development of oral structures. Appropriate interdisciplinary care is needed to successfully address dental abnormalities and prevent dental caries in the primary and the permanent dentitions. A regular dental home plays a key factor in achieving satisfactory oral health goals.

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Impact of therapeutic modalities on craniofacial bones and teeth

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The principal mineralized tissues of the oral cavity include periodontal cementum, dentin, and enamel as well as the bones of the jaw. Unlike cementum or dentin, which can increase in thickness as well as mineralization as a result of pathology, tooth enamel is progressively lost over the life span of an individual. In contrast, bone is a dynamic tissue that undergoes remodeling throughout life in response to various environmental stimuli. While occlusal forces form the primary, physiologic stimuli within the oral cavity, craniofacial bones are susceptible to a variety of pathological factors. Furthermore, therapeutic interventions also can play a significant role in altering the structure and function of craniofacial bones. Recent advances in pathology and molecular medicine have improved our understanding of the mechanisms by which some of these interventions affect mineralized tissue within the oral cavity. This chapter will examine some common agents that can significantly affect tooth enamel and/or the jawbone.

Effects of medical intervention: radiation therapy

Radiation therapy plays important curative, adjuvant, and palliative roles in the treatment of malignant neoplasms. The sequelae of radiation on bone depend on several factors, namely, the age of the patient, total and incremental radiation doses, pattern of application, absorbed dose, size of the irradiated field, beam energy and fractionation, and use of radiation modifiers. The primary effect of radiation on the immature or developing skeleton is the halting of growth. This may be seen with radiation doses as low as 400 cGy; however, perma-

nent damage has been reported only in doses exceeding 1200 cGy (Probert *et al.* 1973). Early changes may be visible as soon as 1–2 months following radiation, but cartilage degeneration and bone atrophy may not be visible for 12 months post radiation treatment. The sexually mature skeleton responds to radiation therapy with osteoradionecrosis (ORN).

Mechanism of action

The basic pathology underlying osteoradionecrosis is atrophy of bone. Several mechanisms contribute to this process, key among which are vascular changes, changes in the bone matrix, and cellular changes (Lyons and Ghazali 2008). Changes in vasculature secondary to irradiation were first described by Ewing (1926). These changes included obliterative endarteritis and periarteritis with cytoplasmic degeneration of the endothelial cells. Later changes involve hyalinization of the media of the artery and obliteration of the lumen due to subintimal fibrosis. Our understanding of the pathophysiology of osteoradionecrosis has undergone several changes over the decades. It was initially thought that radiation above a certain level, injury, and infection were three critical factors for the development of ORN, and this concept prompted the widespread use of antibiotics (Meyer 1970). It was believed that ORN was similar to radiation-induced osteomyelitis in that damage to the cellular structures by high doses of radiation led to the opening of bony channels. Infection of the injured bone led to further destruction, resulting in the clinical signs of ORN. However, Marx (1983) found that 35% of his cases did not have secondary infection. Furthermore, the microbial profile of ORN was significantly different

from that of osteomyelitis; while osteomyelitis of the long bones presented as a mono-infection, mostly by *Staphylococci*, bacteria found in bones affected by ORN appeared to be contaminants (Marx 1983). The hypoxic-hypocellular-hypovascular theory was proposed as an alternative to explain the pathogenesis of ORN and suggests that cell death secondary to irradiation leads to a hypocellular state. When complicated by endarteritis and vascular damage, this leads to hypoxia, which results in a chronic nonhealing wound. This theory led to the widespread use of hyperbaric oxygen in the treatment of ORN. However, hyperbaric oxygen therapy has had limited value as a stand-alone treatment for ORN. The current paradigm of pathogenesis revolves around fibro-atrophic degeneration, similar to that seen in chronic wounds (Dambrain 1993). Histological studies have demonstrated that immediately following radiation, damage to endothelial cells is seen due to both the direct effects of radiation and release of free oxygen radicals. The pro-inflammatory cytokines (e.g., $\text{TNF}\alpha$; interleukin 1, 4, and 6; and $\text{TGF}\beta$) create an acute inflammatory response that further potentiates endothelial damage ultimately resulting in ischemia. Fibroblasts respond to this ischemic state by expressing the myofibroblast phenotype. Myofibroblasts express an aberrant form of extracellular matrix and demonstrate a reduced ability to degrade this matrix. Thus, fibroatrophic degeneration ensues.

Current treatment of ORN includes administration of Pentoxifylline 400 mg twice daily along with 1000 IU of α -Tocopherol (vitamin E; Marx 1983). Pentoxifylline is a $\text{TNF}\alpha$ antagonist that prevents endothelial degeneration and expression of the myofibroblast phenotype and increases collagenase production. Tocopherol is a free radical scavenger that reduces the deleterious effects of reactive oxygen species. While each medication is not capable of demonstrating benefits as a stand-alone therapy, together they appear to decrease the clinical sequelae of ORN.

Bone-sparing drugs: bisphosphonates and RANKL inhibitors

Osteonecrosis of the jaw (ONJ) has been documented in the literature for over a century and has been characterized by bone death as a consequence of a wide variety of systemic and local factors that compromise blood flow (Figure 38.1). Prior to the advent of antibiotics, ONJ was a familiar outcome that was characterized by infection, inflammation, and thrombosis. It resulted from a variety of factors including environmental pollutants, pre-existing diseases, radiotherapy, as well as many popular medications (Hankey 1938). Even though modern medi-



Figure 38.1 ONJ associated with bisphosphonates located on the mandibular lingual mucosa.

cine has prevented the occurrence of massive necrotizing bone infections of the jaws, in the twenty-first century of preventive dental and periodontal care, the use of new and powerful bone-sparing drugs have been associated with the occurrence of ONJ. Despite a growing literature base on the adverse effects of bone-sparing drugs, prevailing uncertainties remain about the etiology, early diagnosis, incidence, and management of an ONJ associated with either bisphosphonates or a RANKL inhibitor.

Although our information about an ONJ in patients using bisphosphonates or a RANKL inhibitor continues to increase, the conventional wisdom about the dental management of individuals is a combination of art and science mixed with ignorance. With the amount of material that is being introduced to the literature monthly on this subject, it would be prudent to consider that additional information further clarifying this disease will continue to accrue. Prior to evaluating the issues related to a drug associated ONJ, it is important to briefly review what bisphosphonates and RANKL inhibitors are, what they are used for, and how they work.

Bisphosphonates: what they are and what they do

In the mid-1960s, inorganic pyrophosphates were found to prevent calcification in body fluid by binding to hydroxyapatite crystals (Fleisch *et al.* 1966). This discovery led investigators to find stable analogs of inorganic pyrophosphates, which are now called *bisphosphonates*. As a family of pyrophosphate analogs, bisphosphonates contain a common chemical configuration: two phosphate groups attached to a central carbon atom that forms a three-dimensional structure (Rogers 2004). This molecular construct enables the molecule to attach to bone (Fournier *et al.* 2002) and disrupts osteoclast function (Murakami *et al.* 1995). In addition to the effects on

bone, they also have anti-invasive (Body 2003), anti-angiogenic (Fournier *et al.* 2002), and antiproliferative properties (Chapurlat & Delmas 2006).

Regarding pharmacokinetics, bisphosphonates are highly polar compounds and as a result are poorly absorbed after oral ingestion (Chapurlat & Delmas 2006). More specifically, the bioavailability of the drug is less than 5% after oral administration (Conte & Guarneri 2004). Because food can reduce absorption, timing of meals is important to enhance the bioavailability of the drug (Chapurlat & Delmas 2006). To increase the amount of bisphosphonates introduced to bone, drug delivery can be accomplished via intravenous administration (Chapurlat & Delmas 2006). Once in the bloodstream, almost the entire dose is either absorbed by the bone or eliminated in urine (Rogers 2004). As a result of their negative charge and chemical structure, bisphosphonates can be retained by the bone for as long as 10 years (Ruggiero *et al.* 2004). When bone remodeling does occur, bisphosphonates are released into the acidic environment of the resorption lacunae where they impede osteoclast action by inhibiting cholesterol biosynthetic pathways, accelerating apoptosis (Kasting & Francis 1992), or disrupting the cell cycle (Murakami *et al.* 1995).

Over time, bisphosphonate structure has been modified to increase efficacy (Licata 2005; Table 38.1). First-generation bisphosphonates (e.g., etidronate) had minimally modified side chains of the pyrophosphate molecule or contained a chlorophenyl group. With the addition of a nitrogen group in the side chain, second-generation bisphosphonate (e.g., alendronate) potency increased by 10- to 100-fold. Third-generation bisphosphonate (e.g., risedronate) potency increased by 10,000 times when a heterocyclic ring containing nitrogen was inserted into the drug molecule.

The value of bisphosphonates resides in their ability to inhibit bone resorption. These drugs are employed for the treatment of osteoclast-mediated bone diseases,

which include osteoporosis, steroid-induced osteoporosis, Paget's disease, tumor-associated osteolysis, multiple myeloma, and malignancies associated with hypercalcemia (Licata 2005). For the prevention of bone metastases, bisphosphonates are important adjuncts commonly used in patients with many types of neoplasms, especially breast and prostate cancer. In dentistry, they have been shown to prevent dental calculus formation (Muhlemann *et al.* 1970) and have shown benefits in modulating host responses in the management of periodontal diseases (Lane 1997; Rocha *et al.* 2004). Bisphosphonates can also have toxic properties and some of the adverse effects include osteomalacia, esophagitis, mild fever, aches, and renal toxicity.

RANKL inhibitors: what they are and what they do

Similar to bisphosphonates, a RANKL inhibitor affects the bone-remodeling process but by an entirely different mechanism. For osteoclastogenesis, the secretion of RANKL (receptor activator of nuclear factor kappa ligand) from osteoblasts is essential. RANKL is a member of the tumor necrosis factor (TNF) family and binds to cell surface receptors known as RANK (receptor activator of nuclear factor kappa) on the pre-osteoclast (Baron *et al.* 2010; Hanada *et al.* 2010). Once bound to RANK, RANKL stimulates the production of numerous proteins that are necessary for osteoclast formation, function, and survival (Baron *et al.* 2010; Hanada *et al.* 2010). A RANKL inhibitor is a protein that can antagonize RANK. Moreover, it will have an affinity for the receptor but will have no efficacy or more specifically will not provoke the biological response leading to osteoclast maturation (Baron *et al.* 2010; Hanada *et al.* 2010).

Currently the only approved RANKL inhibitor is denosumab (Prolia®), a human monoclonal antibody that antagonizes RANK. The pharmacokinetic characteristics of denosumab differ from bisphosphonates in that they are administered by subcutaneous injection and not

Table 38.1 Antiresorptive potency of bisphosphonates currently on US market.

Generic name	Trade name	Manufacturer	Side chain	Relative potency	Administered
Etidronate	Didronel	Procter & Gamble	Short alkyl or halide	1	Orally or intravenously
Tiludronate	Skelide	Sanofi-Aventis	Cyclic chloro	10	Orally
Pamidronate	Aredia	Novartis	Aminoterminal	100	Intravenously
Alendronate	Fosamax	Merck	Aminoterminal	100–1,000	Orally
Risedronate	Actonel	Procter & Gamble	Cyclic nitrogen	1,000–10,000	Orally
Ibandronate	Boniva	Roche	Cyclic nitrogen	1,000–10,000	Orally
Zoledronic acid	Zometa	Novartis	Cyclic nitrogen	≥10,000	Intravenously

incorporated into the bone (Burkiewicz *et al.* 2009). The half-life of denosumab is approximately 32 days, and clearance is by the reticuloendothelial system followed by excretion by the kidneys (Burkiewicz *et al.* 2009).

The value of a RANKL inhibitor resides in the ability to inhibit bone resorption. This drug is employed for the treatment of osteoclast-mediated bone diseases, which include osteoporosis and prevention of skeletal-related events in cancer patients. Denosumab can also have toxic properties, and some of the adverse effects include back, arm, muscle, and leg pain; high cholesterol; allergic reaction; hypocalcemia; bladder infection; and persistent, severe infection (Burkiewicz *et al.* 2009).

Diagnosing an ONJ associated with bone-sparing drugs

The first peer-reviewed report of ONJ associated with a bone-sparing drug was reported in 2004 (Ruggiero *et al.* 2004). Since that time, the absence of a universally accepted case definition, combined with missing historical or clinical patient information, has reduced the quality of many case reports in the literature concerning ONJ. Presently, a confirmed case of ONJ has a clinical presentation that includes soft-tissue swelling and exposed, necrotic bone that has persisted for more than eight weeks (Migliorati *et al.* 2005; American Association of Oral and Maxillofacial Surgeons 2007; Figure 38.1). It is important to note that the eight-week duration of exposed bone in the jaw is necessary to distinguish ONJ from other conditions that exhibit a delayed healing response. To further distinguish ONJ from other maladies, the patient must have taken or be currently using a bone-sparing drug, while other potential confounding conditions (e.g., radiotherapy to the jaws, alcoholism, heavy metal accumulation, heritable prothrombotic tendencies, or corticosteroids) should be eliminated (Table 38.2). Although ONJ may remain asymptomatic for months, it can be associated with localized pain in the

affected area (Migliorati *et al.* 2005). Reported cases are more often identified only in the mandible (65%), while the bone exposure in the maxilla only (26%) or maxilla and mandible (9%) is less common (Woo *et al.* 2006). In the mandible, most lesions were found on the posterior lingual side near the mylohyoid ridge (Woo *et al.* 2006).

Dental management of a patient using bone-sparing drugs

Currently, there is no way to predict which patients who are using bisphosphonates or a RANKL inhibitor are at greatest risk for ONJ, nor are there reliable diagnostic tests that can forecast jaw osteonecrosis. The task force of the American Society for Bone and Mineral Research reviewed various imaging methods for ONJ and has suggested that the most promising modality to detect patients with ONJ is to image bone and soft tissue in individuals using contrast agents combined with magnetic resonance imaging (Khosla 2001). The future evaluation of this modality will need to prove if this is an effective approach, especially in identifying early cases of ONJ. There has also been a recommendation to monitor the levels of bone resorption markers in serum, especially C-terminal telopeptide (CTX), to ascertain when an individual is at risk for ONJ (Marx 2007). Presently, the sensitivity and specificity of CTX for predicting ONJ have not been determined and controlled; randomized clinical trials will be necessary to corroborate the efficacy of this test in detecting ONJ.

Protocols for the management of patients with ONJ have primarily been published for those individuals using bisphosphonates since these drugs have been on the market for a longer period of time than RANKL inhibitors. Since it appears that the occurrence of ONJ associated with a RANKL inhibitor is similar to bisphosphonates (Baron *et al.* 2010), we must assume at this point that management of ONJ for a bisphosphonate or RANKL inhibitor are the same. As such, the recommendations for bisphosphonate-associated ONJ will apply equally for an ONJ that is associated with the RANKL inhibitor. ONJ management protocols have been outlined by task forces from the American Dental Association, the American Association of Oral and Maxillofacial Surgeons (AAOMS; 2007), the American Society for Bone and Mineral Research (Khosla 2001), and the American Academy of Oral Medicine (Migliorati *et al.* 2005). Presently the outcomes for treatment and long-term assessment of treatment and prevention programs from these task forces have not been determined. As a result, many of the suggestions for patient management have been dependent on anecdotal observations and expert opinion.

Table 38.2 Conditions that may present with exposed maxillary or mandibular bone.

Infections leading to osteomyelitis
Osteoradionecrosis
Neuralgia-inducing cavitation osteonecrosis (NICO)
Bone tumors or metastases
Trauma
Herpes zoster infection associated osteonecrosis
Benign sequestration of the lingual plate
Necrotizing ulcerative periodontitis
Excessive absorption of heavy metals

All patients who are going to begin treatment with bone-sparing drugs should receive a dental examination and be informed about the potential adverse oral effects of these drugs (Migliorati *et al.* 2005; Woo *et al.* 2006; AAOMS 2007). Patient management should be directed at reducing future needs of dentoalveolar surgery (Migliorati *et al.* 2005; Woo *et al.* 2006; AAOMS 2007). This means eliminating active sites of infection by periodontal, prosthodontic, and/or endodontic treatment or with appropriate dental extractions. It is also very important to establish meticulous preventive dental regimens for patients (Migliorati *et al.* 2005; Woo *et al.* 2006; AAOMS 2007). Each preventive dental regimen should be customized to patient needs. In general, these regimens should include patient education, oral hygiene home care routines to reduce dental caries and periodontal disease, elimination of habits that can increase dental disease (e.g., smoking and drinking alcohol), and a schedule for routine visits to a dentist. Delaying initiation of bisphosphonate therapy until dental treatment is completed is probably not necessary since there appears to be a three-month window prior to when the first oral pathological outcomes of bisphosphonates were observed (Woo *et al.* 2006).

Patients without ONJ but who are receiving bone-sparing drug therapy should continue to receive dental examinations or receive an examination if a dentist has not seen them prior to the onset of treatment. Information about the potential for adverse oral outcomes needs to be provided, and meticulous preventive dental strategies must continue to be executed. Dental treatment that does not affect the orofacial bone can be executed at any time; however, appropriate nonsurgical and pharmacologic management of dental disease that affects the bone should be attempted prior to dentoalveolar surgery (Edwards *et al.* 2008). If dentoalveolar surgery is necessary, conservative surgical techniques with primary tissue closure should be a prime goal of the dentist (Edwards *et al.* 2008). Postoperative care should include the use of appropriate oral hygiene methods using FDA-approved antimicrobial toothpastes and rinses (Edwards *et al.* 2008).

Controversy exists regarding the need to discontinue bisphosphonate therapy (i.e., a drug holiday) for three months in patients with a putative risk factor or for those who have been on the drug for more than three years. The rationale for the drug holiday is that these patient groups are at higher risk for ONJ and that removal of the drug will potentially lower the chance of inducing ONJ, facilitate soft-tissue healing, and therefore improve outcomes when dentoalveolar surgery is provided (Woo *et al.* 2006; Marx 2007). Presently, there are no data to support or oppose improved dental outcomes with a

drug holiday (Woo *et al.* 2006; Khosla *et al.* 2007). Considering the extended skeletal half-life of these drugs, it is optimistic to consider a significant recovery of bone turnover following such a short period of time without the drug (Woo *et al.* 2006; Khosla *et al.* 2007). In addition to anecdotal reports of improved outcome with a drug holiday, clinical studies are needed to ascertain if drug discontinuation is valuable and to determine the optimal length of a warranted drug withdrawal.

Several clinical conditions may mimic ONJ (Table 38.2); therefore, extensive medical history is beneficial in arriving at a diagnosis. Patients with ONJ who are receiving bisphosphonate therapy have been categorized into four stages that progress from putative prodromal signs and symptoms (stage 0); to asymptomatic patients with exposed bone and no infection (stage 1); to symptomatic patients with exposed bone, infection, and possible purulent drainage (stage 2); and to symptomatic patients with exposed bone, infection, fracture, extraoral fistula, or osteolysis extending to the inferior border (stage 3) (AAOMS 2007). Unless patients are in stage 3, surgical debridement of ONJ has not been encouraged (Carter *et al.* 2005; Zarychanski *et al.* 2006; AAOMS 2007) because it is difficult to find viable bone margins given the broad effects of bisphosphonates in the jaw. When surgical intervention has been attempted, fistulae may develop around flap edges (Zarychanski *et al.* 2006) and enlargement of the necrotic area can occur (Migliorati *et al.* 2005). Symptomatic relief can be obtained with antibiotic therapy and antimicrobial mouth rinses, but this effect appears to be transitory (Zarychanski *et al.* 2006). Removal of bony sequestrum that is mobile and does not impinge on unaffected bone and extraction of symptomatic teeth that are in exposed necrotic bone should be considered (AAOMS 2007). Although a drug holiday has been proposed for those patients who might have to undergo surgical revision of the necrotic site, there is scant documentation to support drug withdrawal (Woo *et al.* 2006; Khosla *et al.* 2007).

Hormones: estrogen

It has been known since the late 1980s that estrogen receptors are expressed by osteoblasts, osteoclasts, and osteocytes. These cells express two types of estrogen receptors—ER α and ER β . While ER α receptors are primarily found in cortical bone, ER β isoforms are found in trabecular bone. It is generally accepted that most of the actions of estrogen on bone cells are mediated by ER α receptors. Estrogen exerts its bone-sparing activity through many mechanisms, both genomic and nongenomic. One fundamental genomic mechanism is by activation of estrogen response elements (EREs) in DNA by

estrogen receptors. When estrogen is bound to the receptor, it dimerizes and binds EREs with great affinity, leading to gene expression. Estrogen receptors can also bind to other DNA-binding proteins such as c-Fos and c-Jun, thereby activating transcription within the cell. Estrogen is capable of signaling through membrane receptors, leading to phosphorylation of the cytoplasmic kinases ERK1 and ERK2. This signaling is an important mechanism by which estrogen inhibits apoptosis of osteoblasts.

Bone turnover is normally mediated by the cooperative actions of osteoclasts and osteoblasts that coexist as an anatomical unit—the basic multicellular unit (BMU). Bone turnover is initiated by the formation of a BMU on the surface of bone. The osteoclasts replace bone-lining cells in areas to be remodeled and create resorption lacunae that remove old bone from the endosteal surface. At the end of the process, osteoclasts undergo apoptosis, terminating the resorptive process and beginning the process of bone apposition, which is carried out by osteoblasts that have been recruited to the resorption lacuna. Estrogen affects bone remodeling by limiting the number of osteoclasts and by blocking formation of new cells from hemopoietic precursors.

Estrogen deficiency increases the activation frequency of BMUs, that is, the number of BMUs formed per unit of time, primarily by increasing the number of osteoclasts within each BMU. This leads to increased depth of each resorption lacuna and increase in cortical porosity. Estrogen deficiency also prolongs the life of each osteoclast by decreasing apoptosis. The basic cytokines required for normal function of osteoclasts are RANKL and M-CSF, both of which are produced by osteoclasts, activated T-cells, and bone stromal cells. Both RANKL and MCP-1 promote the differentiation of osteoclast precursors, activate mature osteoclasts, and increase the fusion of cells to form larger multi-nucleated units. Both cytokines are upregulated by TNF and IL-1, produced by activated T-cells through the NF- κ B and AP-1 pathways. Estrogen suppresses TNF production by T-cells through several pathways, primarily by decreasing T-cell production through an IL-7 dependent pathway. Estrogen also prevents T-cell activation by decreasing the efficiency of antigen presentation and by decreasing production of IL-7 and INF- γ . It has been reported that ovariectomized animals as well as postmenopausal women display greater levels of T-cell-derived TNF due primarily to an increase in the number of TNF-producing cells. This is the currently accepted mechanism for osteoporosis in postmenopausal women (Figure 38.2).

Certain investigations have indicated that postmenopausal osteoporotic women with pre-existing periodontitis are at a higher risk for periodontal bone loss in

response to a similar bacterial challenge (Hankey 1938; Dambrain 1993). These women have also been reported to be at a greater risk for posterior tooth loss (Fleisch *et al.* 1966; Rogers 2004). The risk of implant failure in postmenopausal women with osteoporosis does not appear to be higher than that in non-osteoporotic women (Mellado-Valero *et al.* 2010).

Hormone replacement therapy (HRT) was used to treat postmenopausal osteoporosis as part of the Women's Health Initiative. Both conjugated estrogens as well as estrogen–progesterone combinations were used, however, the trials were discontinued in 2002 when it was found that these therapies increased the risk for breast cancer, pulmonary embolism, stroke, and cardiovascular disease in these women. Selective estrogen receptor modulators (SERMs), also known as *designer estrogen*, have become available as an option for women who cannot be treated with HRT. These drugs react with estrogen receptors through pathways that are significantly different than estrogen pathways. Tamoxifen, a drug that has been used to treat carcinomas of the breast, has demonstrated a decrease in vertebral and hip fractures in routine doses (Cooke *et al.* 2008). However, the drug is associated with a two- to threefold increase in endometrial cancer. Raloxifene is a benzothiophene derivative that has been approved by the FDA for the prevention of postmenopausal osteoporosis. This drug has been shown to decrease bone turnover in a comparable manner to Tamoxifen but is not associated with an increased risk of breast or endometrial cancer (Seeman *et al.* 2006). While it affects the lipid metabolism in a similar fashion to estrogen, unlike estrogen, it does not alter circulating levels of triglycerides.

Hormones: parathyroid hormone

Parathyroid hormone (PTH) has traditionally been regarded as a bone resorptive factor; however, recent evidence indicates that it can be anabolic to bone. The bone anabolic effects of low-dose, intermittently administered PTH were initially discovered by Selye in 1932, whose findings have been confirmed by recent investigations. It is now known that human PTH and its bioactive fragments are capable of stimulating osteoblasts and reversing the signs of osteoporosis. Several mechanisms have been proposed to explain the dual effects of PTH. It has been suggested that PTH exerts its catabolic and anabolic actions through different receptors. Another theory suggests that different messenger systems are activated by PTH, each of which activates a different response. Intermittent injections of PTH stimulate osteogenic activity by triggering bursts of adenyl cyclase activity, leading to cAMP synthesis and triggering

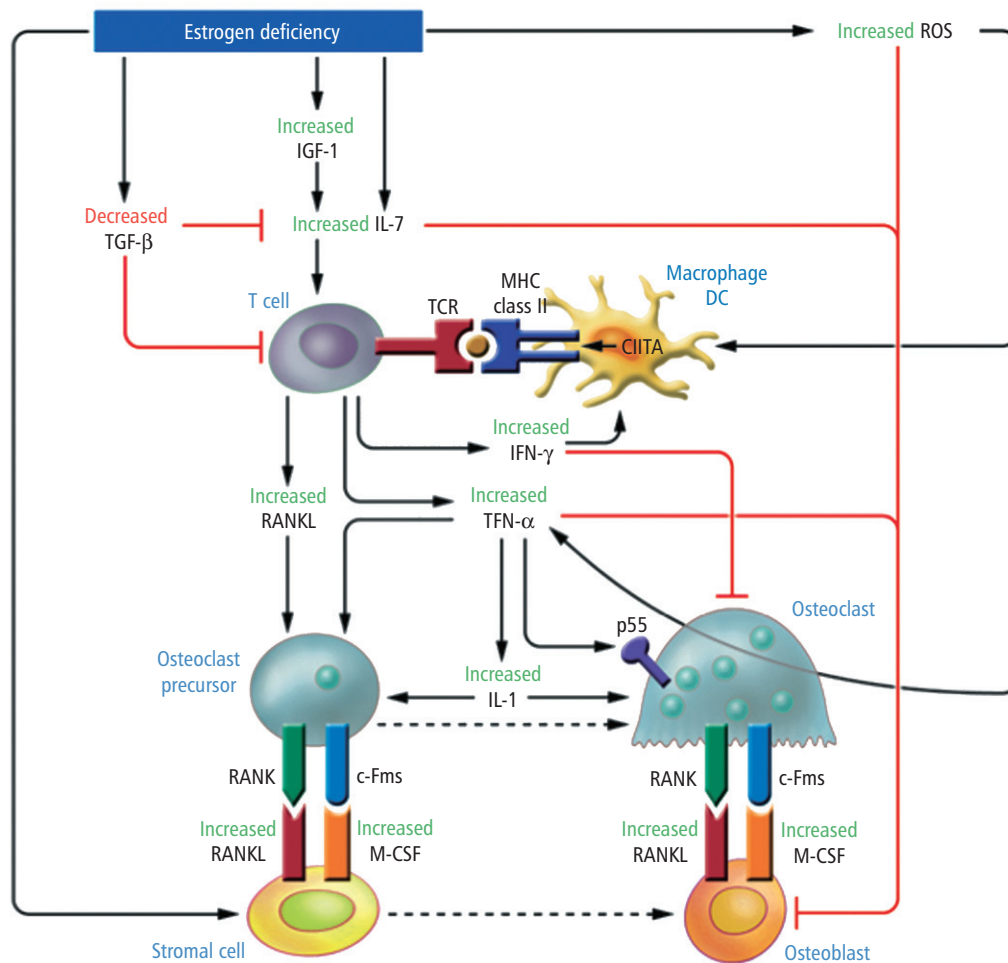


Figure 38.2 Mechanism of action of estrogen deficiency on bone loss (Weitzmann & Pacifici 2006). Estrogen deficiency and bone loss: an inflammatory tale. (Reprinted from Weitzmann and Pacifici, 2006, with permission from the American Society for Clinical Investigation.)

cAMP-dependent protein kinase activity (Whitfield & Morley 1995). It is known that osteoblasts are the primary target of PTH and that intermittent stimulation with PTH leads to the induction of several growth factors in these cells, of which Insulin Growth Factor-I (IGF-I), IGF-II, and TGF are predominant (Dempster *et al.* 1993). Continuous and intermittent administration of PTH also has different effects on RANKL in osteoblasts. Continuous administration increases RANKL expression in osteoblasts and decreases the level of osteoprotegerin, a decoy protein that attenuates the action of RANK, thereby increasing osteoclastic activity (Ruggiero *et al.* 2004). However, intermittent administration has little or no effect on RANKL activity. Animal studies have demonstrated a significant increase in cancellous bone mass, with little or no change in cortical bone (Hock *et al.* 1998). Clinical trials have demonstrated an increase in spine bone mineral density with PTH and a reduction in risk of vertebral fractures in postmeno-

pausal women (Licata 2005). At present, there are some concerns with PTH therapy. The first is the phenomenon of *cortical steal*. It has been demonstrated that PTH increases cancellous bone mass at the expense of cortical bone, leading to questions about the mechanical properties of bone. However, there is no evidence either from animal studies or from human trials to indicate that intermittent PTH administration is not anabolic for cortical bone. Another concern is an increased risk of osteogenic sarcoma that has been demonstrated in rodent models and reported in some clinical trials (Subbiah *et al.* 2010). However, this has not been replicated in primate models, nor is there compelling evidence of increased risk for osteogenic sarcomas in patients with hyperparathyroidism. Further, the bone-anabolic effects of PTH are reversed upon cessation of the medication. Hence, it may be important to combine PTH treatment with anti-resorptive agents such as bisphosphonates (Rocha *et al.* 2004).

Fluorides

Fluorides have received wide attention in dentistry and medicine due to their effects on both teeth and bone. For nearly a century, fluorides have been held to play important roles in the treatment and prevention of dental caries, as well as in the treatment of osteoporosis (Franke 1988). Fluorides are absorbed by the stomach and small intestine through simple diffusion and excreted largely through the kidneys. Most of the ingested fluoride is stored in the mineralized tissues and the levels of this chemical in the tissues increases with age.

Enamel formation occurs as a sequential process that is mediated through epithelial-mesenchymal interactions (Aoba *et al.* 1987). These interactions result in the secretion of tissue-specific proteins, transport of ions, and precipitation of these ions into enamel crystal forms. Ameloblasts perform two critical functions during amelogenesis: they regulate mass transport of ions from systemic to local circulation and vice versa, and they synthesize proteins and proteases (Figure 38.3).

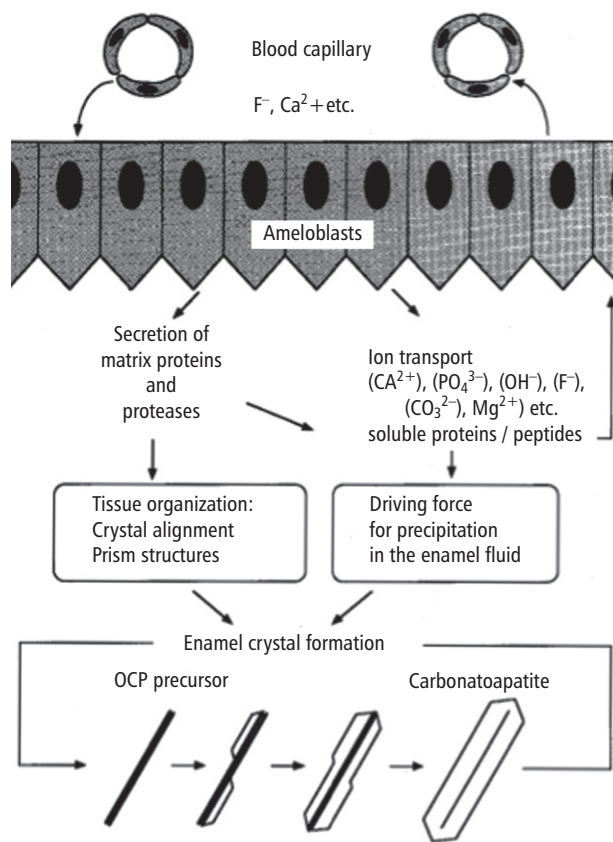


Figure 38.3 Mechanism of action of fluorides on enamel formation. Dental fluorosis: chemistry and biology. (Reprinted from Aoba and Fejerskov, 2002, with permission of SAGE Publications.)

Amelogenin, tuftelins, ameloblastin, and enamelin are the predominant proteins, while enamelysin (MMP-20) and enamel matrix serine proteinase-1 (EMSP-1) are two important proteinases (Fincham and Simmer 1997). During enamel formation, the secreted proteins are responsible for the tissue architecture leading to the formation of enamel prisms and crystal structure orientation within these prism structures. Postsecretory processing of these proteins by the proteinases leads to the formation of soluble cleavage products that allow further mineralization of the newly formed enamel structure. Mineralization is achieved by the formation of octacalcium phosphate crystals (OCP) and the conversion of these crystals into elongated, sheet-like OCP-apatite crystals (Iijima *et al.* 1992). Fluorides play a critical role in this process by driving the precipitation of calcium apatite, stabilizing the precipitated ions accelerating OCP-apatite conversion, and by inducing nucleation and growth of apatite crystals (Aoba *et al.* 1995). Thus, fluorides play an integral role in tooth development (Aoba and Fejerskov 2002). The same mechanisms also play a role in fluoride-induced mineralization of teeth following eruption, thereby decreasing the risk of dental caries. Fluorides increase the resistance to acid demineralization of teeth as well as favor remineralization following caries. Fluoride interacts with hydroxyapatite to form fluoroapatite, which has a stronger crystal structure and lower dissolution rate when compared to hydroxyapatite (Featherstone 1999). This means that enamel dissolution will require acids of greater strength than those present in the oral cavity.

However, fluorosis is a major side effect of excess fluoride ingestion during the developmental, secretory, and maturational stages of enamel formation. Clinically, fluorosis is apparent as white lines on enamel, snow-capped cusps and mottled, pitted tooth surfaces in severe cases. It is important to remember that fluorosis occurs through the same mechanisms that promote tooth mineralization during enamel formation. Excess fluoride leads to alteration of the calcium-dependent activities on the mineralization front of the tooth, and thus there is ineffective cleavage of the structural proteins that form the architecture of the tooth, leading to incomplete or delayed mineralization. Contrary to earlier beliefs, it is now known that the amount of fluorosis in the population increases linearly in proportion to the amount of fluoride ingested, which suggests that there is no minimum safe level below which its effects may not be manifest. The severity of fluorosis depends on the total amount ingested regardless of mode of ingestion (water or supplements). Fluorides have been used therapeutically to increase spinal density in subjects with osteoporosis.

Fluoride stimulates osteoblastic proliferation and increases mineral deposition in bone. Fluorides have been shown to stimulate viability of osteoblasts, increase their mitogenic activity, and stimulate mitochondrial activity in these cells. Fluorides exert their actions by increasing the tyrosine phosphorylation of key signaling proteins in the Ras-Raf-MAPK signal transduction pathway. These osteogenic effects have led to the use of fluorides in the treatment of Paget's disease and in osteoporosis. However, it has been demonstrated that increased skeletal mineral density decreases circulating levels of calcium, which leads to fluoride-associated osteomalacia and may lead to secondary hyperparathyroidism. It has also been shown that although fluorides increase mineral density in the vertebrae and hipbones, there is no reduction in fracture risk. Thus, the use of fluorides in the treatment of osteoporosis is controversial. Fluorides have been used to improve the biocompatibility of titanium in dental implants. The formation of fluoroapatite leads to alkaline phosphatase activity at the bone-implant interface and has been demonstrated to increase bone-implant contact and mineral density and to a decrease in markers of resorption.

Tetracyclines

Tetracyclines were introduced as broad-spectrum antibiotics in 1948. Soon after, in 1956, the first reports of tetracycline-associated tooth discoloration appeared in the literature, and the role of tetracycline in inducing enamel hypoplasia was established. Tetracyclines have an affinity for calcified tissues and chelate calcium, forming tetracycline-calcium orthophosphate complexes. Minocycline, on the other hand, chelates iron along with calcium. Tetracyclines are capable of intrinsically staining both the primary and the permanent dentition if administered during tooth development. This permanent discoloration ranges in color from yellow to gray-brown and is dependent upon the dosage of the medication, duration of administration, stage of tooth development, and type of drug (Cohlan 1977). Initially, this discoloration is fluorescent yellow, but upon exposure to light, it changes to a nonfluorescent brown color due to the formation of an oxidation product (van der Bijl & Pitigoi-Aron 1995). There are also reports of adult-onset tetracycline- and minocycline-induced tooth discoloration (Berger *et al.* 1989). Minocycline-induced discoloration appears as a blue-gray discoloration on the teeth and as green or black discoloration of erupted roots (Poliak *et al.* 1985). Several theories have been proposed to explain this peculiar effect of minocycline. It has been hypothesized that the iron-chelating ability of minocycline leads to the discoloration. The extrinsic theory suggests that high concentrations of minocycline

in the gingival crevicular fluid lead to etching and staining of enamel (Fendrich & Brooke 1984), while the intrinsic theory suggests that collagen found in dentin, cementum, and pulp acts as a storehouse for minocycline, which is then released and oxidized (Bowles & Bokmeyer 1997).

During the 1980s, Golub and colleagues (1991) demonstrated that tetracyclines modified mammalian collagenase and that this activity was independent of their antimicrobial efficacy (Golub *et al.* 1991). It has been shown that tetracyclines exert their effects by inhibiting the activity of matrix metalloproteinases (MMPs) and that certain MMPs (MMP-8, 9, and 13) are highly sensitive to this inhibition, while MMP-1 and 3 are weakly susceptible. Tetracyclines also exert antiresorptive effects through direct action on osteoclasts. Vernillo and colleagues (Vernillo *et al.* 1994) have summarized the evidence on the various mechanisms by which these pharmacological agents exert their bone-protective effects. These mechanisms include:

- Altering intracellular calcium concentration and interacting with the putative calcium receptor
- Decreasing the ruffled border area
- Diminishing acid production
- Diminishing the secretion of lysosomal cysteine proteinases (cathepsins)
- Inducing cell retraction by affecting podosomes
- Inhibiting osteoclast gelatinase activity
- Selectively inhibiting osteoclast ontogeny or development
- Inducing apoptosis or programmed cell death of osteoclasts

Summary

The impact of therapeutic agents can be profound on hard tissues in the oral cavity. Therapeutic doses of radiation, bisphosphonates, and RANKL inhibitors have been shown to cause side effects in the bone of the jaws. In contrast, the decline of estrogen in women during menopause and afterward can induce a loss of jawbone density. Although parathyroid hormone regulates blood calcium levels and may have anabolic actions in the bone, the therapeutic effects and efficacy in the jaw are largely not known at this time. With regard to teeth, especially during development, an excess of fluoride is able to change the composition and appearance of tooth structure, whereas appropriate doses of tetracycline to fight infections can also affect the composition and appearance of tooth structure. Even though there are times when the risks of therapeutic modalities induce side effects, when the benefit of treatment exceeds the risk, continued use of the therapeutic agent must be considered.

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Clinical correlate: osteoradionecrosis of the jaws (ORN)

Nicholas M. Makhoul and Brent B. Ward

Radiotherapy is an important adjunct to definitive surgical treatment of oral cavity squamous cell carcinoma alone or in conjunction with chemotherapy. In addition, radiotherapy maintains a role as a palliative therapy for unresectable cancers of the head and neck. Though a useful modality in the treatment of head and neck cancer, it is not without side effects and complications. Complications of radiation therapy have been described as early or late sequelae. Early manifestations of radiation damage to tissues involve the development of oral mucositis, which can be a debilitating side effect leading to pain, infection, and loss of function. Late complications include xerostomia, loss of taste, trismus, progressive dental and periodontal disease, and, less commonly, osteoradionecrosis of the jaws (ORN).

ORN is a chronic slowly progressive process that is unlike acute insults associated with radiation in the head and neck that tend to resolve within weeks to months of discontinuing therapy. The clinical symptoms associated with ORN can include chronic pain, recurrent and severe infections, facial deformation, sequestration of necrotic bone, and sometimes dysphagia leading to severe malnutrition (Støre & Boysen 2002). Developing ORN is a lifelong risk following radiation therapy, although the majority of cases develop within 6–12 months (Clayman 1997). Due to the complexity of this disorder and complicating factors in its development, there have been many different definitions used to identify patients with ORN. The most encompassing and clinically relevant definition has been described by Wong and colleagues (1997) as “a slow-healing radiation induced ischemic necrosis of bone with associated soft tissue necrosis of varying extent occurring in the absence of local primary tumor necrosis, recurrence or metastatic disease.”

Factors that have been postulated to predispose patients to ORN are tumor size and location, radiation dose, local trauma, dental extractions, infection, nutritional deficient status, and host compromise (Marx 1983; Wong *et al.* 1997). In a retrospective study by Reuther and colleagues (2003) that included 830 irradiated head and neck tumor patients, there was an overall incidence of 8.2%, with the most commonly affected area being the body of the mandible (Reuther *et al.* 2003). This study also found advanced tumors, segmental resections, and pre- or postradiation tooth extractions to be the most significant risk factors (Reuther *et al.* 2003). A retrospective cohort study of 82 patients carried out by Goldwaser and colleagues (2007) looking at the risk factor assessments for development of ORN showed a strong correlation between dose of radiation and incidence of ORN. There was a statistically significant increase in cases with doses of radiation greater than 66 Gy. Nutritional status was also found to be an important predictive factor as exhibited by patients with higher Body Mass Index (BMI > 25 < 30) that was associated with a decreased rate of ORN (Goldwaser *et al.* 2007). An important consideration in these historical data of incidence and associated risks is the development of a more sophisticated delivery of radiation used by most centers today in which computer modeling and multiple port delivery limit the radiation to structures distant to the tumor bed. Using these technologies, the rate of ORN has dramatically decreased (Studier *et al.* 2006; Chang *et al.* 2007; Ahmed *et al.* 2009). Regardless, when ORN does develop, it presents as a challenging entity with regard to treatment.

One of the most well-known and well-studied risk factors associated with ORN is trauma. Trauma is usually

due to dental extractions, denture trauma, or iatrogenic trauma from surgical procedures. Reuther and colleagues (2003) reported that the patients diagnosed with ORN had periodontal problems ranging from mild gingivitis to severe periodontitis. Twenty-four percent of ORN patients had teeth extracted prior to radiation, and 26% had teeth extracted after radiotherapy (Reuther *et al.* 2003). Although the presence of dental and periodontal disease has a strong correlation with ORN, spontaneous ORN is also well known as seen in edentulous patients, which makes the pathophysiology of ORN more complicated than simply an infectious process in an irradiated host.

The pathophysiology of ORN has been widely debated and was first described by Meyer (1970) as a traumatic injury to bone and soft tissue that is vascularly compromised by radiation leading to a secondary infection. This theory was later challenged by Marx (1983) who studied 26 cases of ORN; he stained resections for microorganisms and found that although superficial infection was present in the samples, there were no deep-seated organisms, thereby refuting the ORN infectious disease theory. Histologic studies from these specimens showed endothelial death, hyalinization and thrombosis of vessels with fibrotic periosteum, and decreased numbers of osteocytes and osteoblasts leading to the *three-H hypothesis*—hypovascularity, hypoxia, and hypocellularity—as the pathophysiologic cause of ORN (Marx 1983). The current mechanism, which is believed to be the cause of ORN, is a radiation-induced fibroatrophic mechanism that is due to the formation of free radicals leading to endothelial dysfunction, inflammation, microvascular thrombosis, fibrosis, and finally dysfunctional remodeling leading to necrosis secondary to depletion osteogenic cell lines (Delanian & Lefaix 2004). Given the complexity of changes that are induced by radiation, the exact details of this disease have not yet been elucidated and remain an active field of research.

Case

This clinical case report presents a patient diagnosed with ORN and discusses the diagnostic considerations and one potential treatment modality. A 77-year-old male presented to the Department of Oral and Maxillofacial Surgery at the University of Michigan Hospital with a chief complaint of a chronic draining fistula in his left neck following unsuccessful treatment of ORN. A review of the history of his present illness revealed that he had been treated for left parotid gland squamous cell carcinoma with a left parotidectomy, left radical neck dissection, and postoperative radiation therapy seven

years earlier. It was unclear what dose of radiation or delivery method he received. In 2008, a tooth in the left posterior mandible was extracted after which he developed ORN, which ultimately led to a pathologic fracture to the area. In October 2008, he was taken to the operating room at an outside institution, given hyperbaric oxygen therapy, and underwent surgical debridement of his left mandible along with placement of a reconstruction plate and recombinant bone morphogenetic protein (BMP). Subsequent to this failed treatment, he presented to our clinic with cutaneous exposure of his reconstruction plate and residual mandible. He also had an oral cutaneous fistula involving the left side of his face and mouth.

The patient's medical history included chronic lymphocytic leukemia, deep venous thrombosis, basal cell carcinomas, hypercholesterolemia, hypertension, glaucoma, and coronary artery disease. His medications included ASA, lisinopril, atenolol, lasix, Zocor, and multivitamins. He denied smoking and had a social history of alcohol use.

Panoramic examination (Figure 39.1) revealed a reconstruction plate in the left mandible and moth-eaten appearance of the left body of the mandible. Intraorally he had a significant amount of trismus with no visible leukoplakic, erythroplakic, or ulcerative lesions or masses. His neck was without masses or lymphadenopathy. The left side of his neck showed evidence of prior

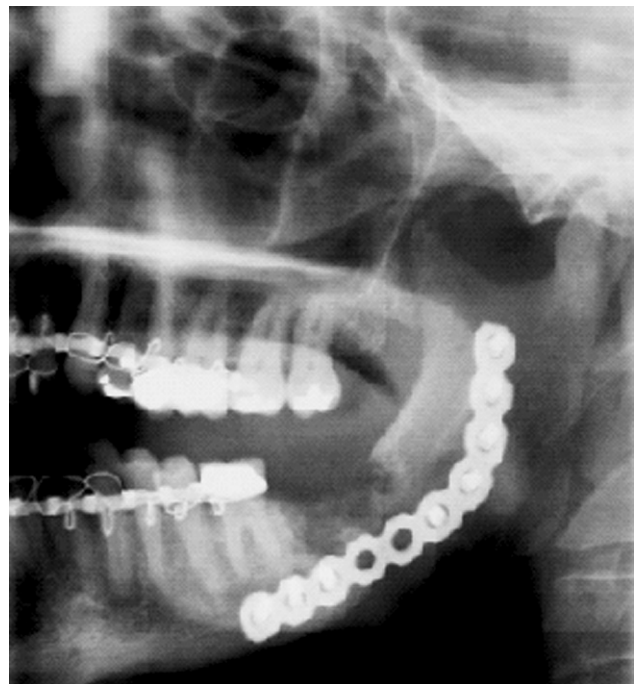


Figure 39.1 Panorex on presentation.



Figure 39.2 Exposure of bone and hardware through left neck.



Figure 39.3 Debridement of necrotic bone.

radical neck dissection and radiation changes to the skin. The reconstruction plate and mandibular bone can be seen through the skin on the left side of the neck (Figure 39.2).

Treatment included hardware removal and debridement or excision of necrotic bone from the left mandible (Figures 39.3 and 39.4). To replace soft tissue over the residual mandible and to close the oral cutaneous fistula, a temporoparietal galea flap (vascularized local soft tissue flap) was elevated with a 6 × 2 cm skin flap and

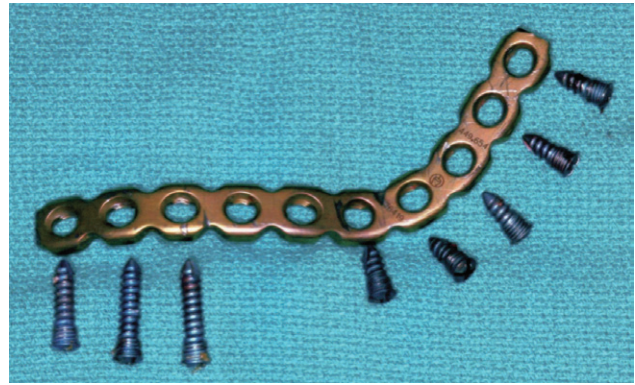


Figure 39.4 Hardware removed.



Figure 39.5 Elevation of the temporoparietal galea flap.



Figure 39.6 Tunneling of flap to reach defect in neck.

tunneled under the left face to the inferior border of the mandible (Figures 39.4 and 39.5). The skin island was then inset to the defect in the face with sutures, and the scalp was closed in a layered fashion (Figure 39.6). He was followed closely during healing and had an uneventful postoperative period. His wounds were closed, and



Figure 39.7 Closure of neck and scalp.



Figure 39.9 Two months postoperative close-up of the closed wound.



Figure 39.8 Two months postoperative.

there was no exposure of bone or fistula present on follow-up examinations (Figures 39.7, 39.8, and 39.9).

Discussion

The diagnosis of ORN is primarily based on exposure of bone with a correlating clinical history for radiation. Although many cases of ORN remain asymptomatic, some patients may have symptoms such as pain, dysesthesia, fetor oris, dysgeusia, and impaction of food in the exposed area (Chrcanovic *et al.* 2010a). In many cases, such as the one presented, ORN is associated with func-

tional problems and disfigurement. In the presented patient, the clinical exam and medical history alluded to the diagnosis of ORN. Corroborative diagnostic techniques including radiographs, computed tomography (CT) scans, magnetic resonance imaging (MRI), Doppler ultrasound, nuclear medicine scans, and infrared spectroscopy have all been used to detail the extent of the process (Chrcanovic *et al.* 2010a). The appearance of ORN on panorex exams usually consists of areas of mixed radio-opaque and radiolucent lesions; however, there must be significant change in the mineralized matrix in order to identify affected areas. Therefore, plain radiographs tend to underestimate the degree of involvement. CT scans have similar limitations but can yield a higher detailed image of bony involvement. Fujita and colleagues have described the use of an MRI to suggest fibrosis of bone marrow and predict the extent of ORN after radiation (Fujita *et al.* 1991). Radionuclide bone scanning with technetium methylene diphosphate has been used to show pathologic changes in bone that would not otherwise be seen on CT or plain radiography; this reflects changes in osteoblastic activity and blood flow but does not offer adequate resolution of the affected areas (Chrcanovic *et al.* 2010a). Even with our advancements in diagnostic imaging, there is no perfect way to detect ORN-affected areas and, therefore, one must rely on clinical and intraoperative examination of tissues in order to treat patients appropriately.

Prevention of ORN has been largely allotted to dental pretreatment evaluation and aggressive elimination of dental disease before initiation of high-dose radiation (Chrcanovic *et al.* 2010b). Periodontal tissue destruction from poor oral hygiene and smoking are major risk factors and can accelerate the development of ORN (Katsura *et al.* 2008). Some institutions that treat head and neck cancer have routine pre-radiation dental “clear-

ances” to aid in avoidance of developing complications both during and after radiation therapy. If pre-radiation extractions are planned, a period of 2–3 weeks pre-therapy has been advocated to allow the time needed for osteoid to form in sockets and epithelialization of the mucosa to occur (Epstein *et al.* 1987; Marx & Johnson 1987). Recent data suggest that this practice may actually increase the risk of ORN, but prospective randomized data are lacking to support this conclusion (Chang *et al.* 2007).

If postradiation extractions are necessary, conservative techniques with minimal trauma to hard and soft tissues should be used. Adjunctive use of long-term antibiotics and the use of HBO surrounding dental extractions for prophylaxis against development of ORN have been extensively described, but there is a lack of robust data and controversy exists regarding their use.

Surgical management of ORN varies from conservative transoral debridement of the sequesterum of bone to major resection and reconstruction with composite osteomyocutaneous microvascular free tissue transfer. In the case of the patient presented, the approach was to remove necrotic bone and hardware, which can be a continuous source of infection, and replace it with vascularized soft tissue from the galea to promote healing and allow closure of the fistula. Resection of bone in a case such as this involves removal until bleeding viable bone is encountered. Depending on the amount of bone resected and the preference of the patient, reconstruction can involve bone transfer and mandibular continuity or soft-tissue coverage alone. For patients with significant trismus, soft tissue alone may have the advantage of increasing maximal incisal opening since bone continuity is not reestablished. The use of a soft tissue-only flap in this case was for this specific purpose.

Conclusion

ORN can be a debilitating disease process. It frequently requires surgical intervention to introduce viable vascularized tissue to areas where bone has become exposed and prior radiation has compromised the body’s ability to heal as in the case presented in this chapter.

Summary

Radiation therapy is used in the treatment of head and neck cancer patients as primary, adjunctive, or palliative treatment. Its use has enhanced successful outcomes for patients with head and neck cancer. Some of the side effects of radiation include mucositis, loss of taste, periodontal disease, dental caries, and osteoradionecrosis (ORN) of the jaws. ORN, in particular, can be a debilitating and deforming process that is believed to result from

free radical damage leading to hypovascular, hypocellular, and architectural changes to the affected bone. ORN of the jaws is primarily limited to the mandible where it has been frequently associated with complications from trauma such as tooth extractions and osseous procedures within the radiation field.

Initial treatment for individuals who develop ORN includes conservative management with antibiotics and minimal debridements. Definitive treatment of advanced cases of ORN entails radical resection and reconstruction with vascularized soft- or hard-tissue flaps to restore function and quality of life.

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